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Pulmonary vascular reactivity in the isolated perfused lungs of normoxic and hypoxic rats

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PULMONARY VASCULAR REACTIVITY IN THE ISOLATED PERFUSED LUNGS OF NORMOXIC AND HYPOXIC RATS

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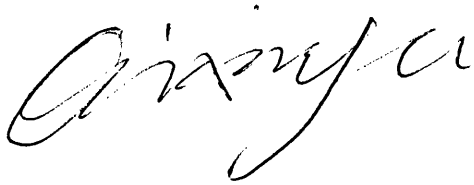

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SUMMARY

Chronic hypoxia (CH) induces pulmonary hypertension and pulmonary vascular remodelling. Accompanying this, the pulmonary vascular bed becomes hyper-reactive to vasoconstrictors, which was demonstrated in the isolated perfused rat lung. Agonists such as phenylephrine (PHE), KCl, angiotensin II (Ang II), noradrenaline (NA) and U46619 all produced enhanced responses, suggesting a common mechanism was responsible.

As in CH lungs endothelin (ET) synthesis is increased, its role in pulmonary vascular hyper-reactivity was studied. ET-1 sensitized pulmonary vasculature to PHE and Ang II in the normoxic lungs via ET_A and ET_B receptors. PKC and the Na^+/H^+ exchanger are not involved in this sensitization. However ET-1 did not potentiate vasoconstrictor responses to KCl. Furthermore in CH lungs ET receptor antagonists and an ET converting enzyme inhibitor did not affect the potentiated vasoconstrictor responses seen in CH lungs. Thus a role for ET-1 to pulmonary vascular hyper-reactivity during CH was not found.

Time-dependent study of vasoconstrictor responses and pulmonary vascular remodeling in CH showed that the increase in responses to the vasoconstrictors Ang II and KCl during hypoxia (3 weeks) and recovery (3 weeks) parallels pulmonary vascular remodelling. However the decline of PHE-induced vasoconstrictor responses during recovery is faster than regression of pulmonary vascular hypertrophy. These differences suggest that another mechanism apart from an increase in the number of pulmonary smooth muscle cells must be involved in the potentiated responses to PHE.

CONTENTS

CHAPTER ONE

INTRODUCTION

1.1 Hypoxic pulmonary hypertension

Alveolar hypoxia (hypoxia) is defined as a decrease of the partial pressure of oxygen (PO_2) in the environment. Exposure to hypoxia provokes a series of modifications in the human body, including the pulmonary circulation. Pulmonary vasculature is very sensitive to changes of alveolar PO_2. An interesting phenomenon is that the pulmonary circulation reacts to hypoxia by vasoconstriction, but the systemic vessels usually dilate in response to hypoxia (Brij and Peacock, 1998). Hypoxic pulmonary vasoconstriction (HPV) is important in keeping an optimal ventilation/perfusion ratio and preventing systemic hypoxaemia. However, in chronic hypoxia (CH), HPV is persistent and followed by the muscularization of the pulmonary vessels, which results in the development of pulmonary hypertension.

The pulmonary circulation is a capacitive and low resistance system. Small changes in pressure induce large changes in arterial diameter. There is little active vasomotor tone, arterial pressure being around 15 mmHg in normal condition in humans. In pulmonary hypertension, systolic pulmonary artery pressure may exceed 25 mmHg (Bishop *et al.*, 1995; Egermayer *et al.*, 1999).

Clinically, most pulmonary hypertension occurs in people living at high altitude or with chronic obstructive pulmonary diseases due to hypoxia. Primary pulmonary hypertension is a condition associated with progressive elevation in the pulmonary pressure, for which no underlying cause can be found. In newborn babies, a dramatic transition of the

pulmonary circulation from a high resistance state *in utero* to a low-resistance state occurs within minutes after birth. If the pulmonary vascular resistance fails to decrease, this will lead to pulmonary hypertension, a condition known as persistent pulmonary hypertension of the newborn. Perinatal hypoxia is thought to be the reason for the persistant pulmonary hypertension of the newborn (Abman, 1999). Anorexigens such as fenfluramine can induce pulmonary hypertension, which may be due to increased 5-hydroxytryptamine (5-HT) levels in the lungs (Valodia & Syce, 2000). Other reasons for pulmonary hypertension are congenital systemic to pulmonary shunts, pulmonary embolism and sarcoidosis (Galie & Torbicki, 2001). Exposure to hypoxia is a common model for experimental study of pulmonary hypertension. Monocrotaline-induced pulmonary hypertension is another useful animal model for study of pulmonary hypertension (Ito *et al.*, 1988; Wanstall & O'Donnell, 1990). Monocrotaline, a pyrrolizidine plant alkaloid, is metabolized in the liver into an active pyrrole form, which causes pulmonary endothelial damage leading to pulmonary edema, protein leakage and cellular filtration and then gradually induces pulmonary hypertension (Mathew & Altura, 1990; Wilson *et al.*, 1992). Although the initial factors may differ widely between different forms of pulmonary hypertension, chronic pulmonary hypertension commonly results in decreased pulmonary vascular compliance, progressive elevation in pulmonary artery pressure, increase in pulmonary resistance, right ventricular hypertrophy and ultimately heart failure (Giaid *et al.*, 1993; Salvi, 1999).

1.2 HPV

HPV, first described by Von Euler and Liljestrand in 1946, is an important physiological process involved in the matching of regional pulmonary blood flow to regional ventilation. Alveolar hypoxia diverts blood flow from poorly ventilated alveoli to the better-ventilated regions, thereby optimizing ventilation/perfusion ratio matching and maintaining an adequate systemic PO_2. In the foetus, this HPV serves to increase pulmonary vascular resistance and divert blood flow from the pulmonary circulation into the systemic circulation through the ductus arteriosus. The foetal pulmonary circulation therefore only receives up to 10% of the cardiac output (Sansoucie & Cavaliere, 1997). After birth, HPV is an important negative feedback mechanism required for ventilation-perfusion matching. HPV has been demonstrated in a variety of species, e.g. rat, rabbit, cat, pig, calf, sheep and human (reviewed by Wadsworth, 1994).

The pulmonary artery tree is a complex system due to regional differences in structure and functions (Kemp *et al.*, 1997). There are 15 orders of arteries between the main pulmonary artery and the capillaries in human lungs, which are divided into three descending segments, namely, elastic arteries, muscular arteries and arterioles (Sasaki *et al.*, 1995; Huang *et al.*, 1996). The main pulmonary artery and primary branches of pulmonary arteries are elastic arteries, which are essentially similar to large systemic conductive arteries, e.g. aorta. These pulmonary arteries have well-developed internal and external elastic laminae and a less distinct medial layer than that of systemic arteries (reviewed by Maclean *et al.*, 2000). Distal to the elastic pulmonary arteries, pulmonary

arteries taper to the muscular arteries, defined by diameters from 100 μm to 1mm. Pulmonary arteries < 100 μm in rats tend to have no media or only a scattering of smooth muscle cells, and are called arterioles (Sasaki *et al.*, 1995; MacLean, 1999a; Salvi, 1999). The last order of the pulmonary artery tree is 20 μm in diameter in humans and 13 μm in diameter in rats (Huang *et al.*, 1996). HPV occurs at different levels of the pulmonary artery tree, in both the large and small arteries (Yuan *et al.*, 1990). However, the small muscular pulmonary arteries and arterioles of the pulmonary artery tree are thought to be the major site of HPV when considering the increase of pulmonary resistance (MacLean, 1999a; Salvi, 1999). The study of isolated feline pulmonary smooth muscle cells has shown the importance of the small pulmonary artery for HPV because the smooth muscle cells from small pulmonary arteries exhibited significant contraction during hypoxia, whereas the cells from large pulmonary arteries did not contract much (Madden *et al.*, 1992). Using an X-ray TV system, the internal diameters of the feline pulmonary arteries (100 - 600 μm) were monitored. The percentage reduction of the internal diameter during acute hypoxia was greater in pulmonary arteries of 200 – 300 μm internal diameters than in large pulmonary arteries up to 600 μm (Shirai *et al.*, 1986).

There are two main stages to HPV demonstrated in rat pulmonary arterial rings: a rapid transient constriction stage, called phase 1; and a slow, long-sustained stage, called phase 2. Phase 1 occurs independently of the pulmonary endothelium and is thought to result from membrane depolarization of the pulmonary smooth muscle cells; phase 2 is endothelium-dependent, requiring the release of vasoconstrictors from the endothelial cells (Bennie *et al.*, 1991; Robertson *et al.*, 1995). In contrast to pulmonary vasculature,

responses to hypoxia in systemic arteries (e.g. aorta, bronchial artery, mesenteric artery, coronary artery and femoral artery) are different from the pulmonary artery (Leach *et al.*, 2000; Liu *et al.*, 2001), a complete relaxation occurs after a small initial increase in tension. Therefore, an intrinsic mechanism for HPV exists specifically in pulmonary vasculature.

The exposure to hypoxia causes potassium channel (K^+ channel) inhibition, inducing membrane depolarization in the pulmonary arterial smooth muscle cells, but not in the vascular smooth muscle cells from systemic arteries, e.g. renal artery and mesenteric artery (Karamsetty *et al.*, 1995). That is because there are different K^+ channel distributions in pulmonary and systemic arterial smooth muscle cells (Weir & Archer, 1995; Brij & Peacock, 1998). In the systemic arteries, ATP sensitive K^+ (K_{ATP}) channels are opened during hypoxia, thereby hyperpolarizing the membrane and inhibiting calcium (Ca^{2+}) influx (Brij & Peacock, 1998). Voltage-dependent K^+ (K_v) channels are responsible for the membrane depolarization in pulmonary arterial smooth muscle cells during hypoxia (Barman, 1998). It has been demonstrated that mRNA expression of K_v channel α-subunits was decreased in pulmonary smooth muscle cells during hypoxia but not in mesenteric artery smooth muscle cells (Platoshyn *et al.*, 2001). Three sub-types of K_v channel α-subunit, namely $K_{v2.1}$, $K_{v1.5}$ and $K_{v3.1}$ are responsible for the membrane depolarization in pulmonary smooth muscle cells during hypoxia (Archer *et al.*, 1998).

Membrane depolarization brings the membrane potential to a level where L-type Ca^{2+} channels can be opened, leading to increased Ca^{2+} influx into the vascular smooth muscle

cells (Post *et al.*, 1992; Evans *et al.*, 1998; Doi *et al.*, 2000b). Increase of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in pulmonary vascular smooth muscle cells is essential in the development and maintenance of pulmonary hypertension by mediating pulmonary vasoconstriction and stimulating pulmonary vascular smooth muscle proliferation (Platoshyn *et al.*, 2001). The Ca^{2+} channel agonist BAY K8644 potentiated HPV and nifedipine, a Ca^{2+} channel blocker inhibited it in both resting pulmonary rings and those pre-stimulated with either phenylephrine (PHE) or angiotensin II (Ang II), which suggests that HPV involves Ca^{2+} entry through voltage-dependent Ca^{2+} channels (Rodman *et al.*, 1989). Ca^{2+} influx is the major source of depolarization-induced increase of $[Ca^{2+}]_i$ rather than Ca^{2+} release from the internal store, even though there is evidence that Ca^{2+} release is also involved in the membrane depoplarization of smooth muscle cells (Erne & Hermsmeyer, 1991; Barman, 1998).

It is universally accepted that hypoxia induces K^+ channel inhibition on the membrane of pulmonary vascular smooth muscle cells. However, it is uncertain how the smooth muscle cells sense and transmit the changes of PO_2 to the K^+ channels on the cell's membrane. It is presumed that an O_2 sensor exists in pulmonary smooth muscle cells. K_v channels could be the O_2 sensor by themselves, which directly detect the change of O_2 tension (Coppock *et al.*, 2001). Another candidate might be the electron transport chain in mitochondria, because inhibitors of the proximal region of the electron transport chain (rotenone, myxothaizol and diphenyleneiodonium) abolished HPV in isolated perfused lungs and cultured pulmonary smooth muscle cells, without affecting the pulmonary vasoconstrictor responses to U46619 (Archer *et al.*, 1993).

Membrane depolarization could be an initial reaction to hypoxia in pulmonary vasculature. If hypoxia is prolonged, a persistent elevation in pulmonary arterial pressure appears which is not immediately or totally correctable upon improvement in oxygen concentration and cannot be explained solely by membrane depolarization. Locally-released vascular mediators are important in the regulation of pulmonary vascular tone in CH (Raj *et al.*, 1992). Among such mediators endothelin-1 (ET-1), as a potent vasoconstrictor, is strongly implicated in hypoxic pulmonary hypertension (Michael & Markewitz, 1996; MacLean, 1999a). The role of mediators in the regulation of pulmonary vascular tone will be discussed later.

1.3 Pulmonary vascular remodelling CH

HPV is the initial event in the pulmonary circulation during hypoxia, followed by pulmonary vascular remodelling; both of them contribute to pulmonary hypertension (MacLean, 1999a). Hypertrophy and hyperplasia of the pulmonary arterial smooth muscle cells and an increase of matrix in muscular pulmonary arteries are known as pulmonary vascular remodelling (Rabinovitch *et al.*, 1979; Kourembanas & Bernfield, 1994). Smooth muscle cells also extend into distal, previously partly- or non-muscular arterioles (Hislop & Reid, 1976). The elastic laminae also increase in pulmonary arteries during CH (Liu, 1997). Hypoxia also increases intimal thickness by causing hypertrophy and hyperplasia in both the endothelial and subendothelial layers (Durmowicz & Stenmark, 1999). The increases of wall thickness in CH were found in all size ranges of pulmonary artery (Hislop & Reid, 1976). The cellular and structural changes observed in pulmonary hypertension vary significantly, depending on the duration and degree of hypoxic exposure (Durmowicz & Stenmark, 1999). The increase of the vascular wall thickness observed in small pulmonary arteries in mice happens as early as after 2 days of hypoxia (Quinlan *et al.*, 2000). Right ventricular hypertrophy, another parameter of pulmonary vascular remodelling, appeared after 5 days of exposure to hypoxia (Hislop & Reid, 1976). Vascular remodelling also occurs in pulmonary veins during hypoxia. A significant but smaller degree of medial thickness was observed in small pulmonary veins compared to the arteries (Takahashi *et al.*, 2001).

Pulmonary vascular remodelling is reversible and the regression process depends on the

duration of exposure to hypoxia. 10 days hypoxia (10% O_2) significantly increased the pulmonary arterial wall thickness compared to the controls. In recovery rats, which were returned to normoxic conditions, the pulmonary arterial wall thickness decreased continuously and reached normal levels after 30 days (Liu, 1997). Rats under hypoxia (11% O_2) for 4 weeks displayed a significant increase in medial thickness of the pulmonary artery. After 4 weeks recovery, the rats still exhibited the same increase in pulmonary medial thickness as hypoxic rats (Dingemans & Wagenvoort 1978). In other words, pulmonary vascular remodelling from 4 week- hypoxic rats needs more than 4 weeks to recover to the normoxic level.

ET-1, Ang II, 5-HT have all been demonstrated to stimulate proliferation of pulmonary smooth muscle cells (Jeffery & Wanstall, 1999; Eddahibi *et al.*, 2000; MacLean *et al.*, 2000). Therefore, these mediators might contribute to pulmonary vascular remodelling during CH.

1.4 Pulmonary vascular hyper-reactivity during CH

CH induces not only pulmonary vasoconstriction and pulmonary remodelling but also pulmonary vascular hyper-reactivity. The earliest report of pulmonary vascular hyper-reactivity during CH is to Ang II, prostaglandin $F_{2\alpha}$ and noradrenaline (NA) by McMurtry (1978), using isolated perfused rat lungs. Later, Emery *et al.*, (1981) reported that Ang II- and ATP-induced vasoconstrictor responses were potentiated in perfused lungs of CH rats. The threshold dose-responses to Ang II and ATP in control and CH rats were not changed, only at the higher doses were vasoconstrictor responses significantly enhanced in CH lungs. Until now, pulmonary vascular hyper-reactivity during CH has not been thoroughly investigated. Although it is generally accepted that vasoconstrictors produce higher responses in lungs from CH animals, vasoconstrictor responses in pulmonary artery rings are varied. For example, in CH rats ET-1-induced contractile responses were potentiated in the pulmonary resistance arteries, but reduced in main pulmonary arteries (McCulloch *et al.*, 1998; Lal *et al.*, 1999a).

The mechanism of pulmonary vascular hyper-reactivity is not clear. It could be due to 1) pulmonary vascular remodelling; 2) increase in receptor linked second messenger processes; 3) receptor 'cross-talk' amplifying effects of one agonist; 4) sensitisation of contractile elements to Ca^{2+}; 5) membrane depolarization. The possible contributions of each of these mechanisms to vascular hyper-reactivity are discussed below.

Pulmonary vasculature shows vasoconstriction during the early stage of hypoxic

pulmonary hypertension, and this is gradually supplanted by progressive structural changes, so called 'muscularization' (Meyrick & Perkett, 1989). The pulmonary vascular hyper-reactivity is thought to be due to this pulmonary vascular remodelling. Because this new muscle encroaches on the vascular lumen and makes it narrower, a given degree of muscle shortening which reduces the vessel circumference, will cause a larger percentage reduction in radius and a higher increase in vascular pressure (Emery *et al.*, 1981).

Certainly hyper-reactivity appears confined to pulmonary vessels as in ovine uterine artery, vasoconstrictor responses to NA were attenuated in CH (Hu *et al.*, 1999). This was associated with a decrease of IP_3 binding affinity to its receptors was found during CH. In pulmonary artery, an increase in receptor-linked second messenger processes during CH appears to contribute to vascular hyper-reactivity

MacLean *et al.* (1994a) have reported that sumatriptan (a 5-$HT_{1D/1B}$-receptor agonist) fails to induce contraction of bovine pulmonary arteries in the absence of tone. However, under pre-contraction with U46619, sumatriptan is a potent vasoconstrictor. This might be another possibility to explain pulmonary vascular hyper-reacitivity during CH in which pulmonary hypertension unmasks receptor-mediated vasoconstriction.

Agonist (e.g. NA and Ang II) -induced vasoconstrictor responses in certain vessels can be potentiated in the presence of a threshold concentration of a second agonist (e.g. ET-1) (Yang *et al.*, 1990; Nakayama *et al.*, 1991; Wong-Dusting *et al.*, 1991). This could be due

to crosstalk between agonists acting via different G protein-coupled receptors, which amplify the signals (MacLean, 1999b). Or, the second agonist, e.g. ET-1 could increase the sensitivity of the contractile apparatus to Ca^{2+} in the vascular smooth muscle cells (Ohanian *et al.*, 1997; Evans *et al.* 1999). In the mesenteric artery of normotensive rats, ET-1 potentiated NA-induced vasoconstrictor responses and this potentiation effect was prevented by BQ788, a selective ET_B receptor antagonist. Furthermore, in mesenteric arteries of hypertensive rats, vasoconstrictor responses to NA were potentiated compared to normotensive rats, and this potentiation effect was also attenuated by BQ788 (Kita *et al.*, 1998). Thus in these situations where vascular production of ET-1 is known to increase (Schiffrin, 1999), vascular hyper-reactivity may result.

The vasoconstrictor responses to 5-HT were also potentiated in isolated perfused canine lungs during acute hypoxia induced in canine lung lobes ventilated by 95% N_2 gas mixture. In these acute experiments cromakalim, a K_{ATP} channel opener, attenuated hypoxia-induced potentiation of vasoconstrictor responses to 5-HT (Barman, 1998). It is reported that hypoxia depolarises the membrane potenatial via K^+ channel (Archer *et al.*, 1998). The membrane depolarisation brings the membrane potential closer to the threshold for activation of L-type Ca^{2+} channels (Yuan *et al.*, 1990; Evans *et al.*, 1998). Vasoconstrictors can then induce extracellular Ca^{2+} influx more easily during CH. Therefore it is possible that K^+ channel inhibition plays a key role in pulmonary vascular hyper-reactivity during CH.

1.5 Innervation of pulmonary vasculature and hypoxic pulmonary hypertension

The pulmonary vessels are innervated by sympathetic, parasympathetic (vagal) and nonadrenergic, noncholinergic nerves (El-Bermani, 1978). The density of sympathetic nerve fibers is extensively distributed around extrapulmonary arteries; the sympathetic innervation in the intrapulmonary arteries varies between species (Zorychta & Richardson, 1992; Barnes & Liu, 1995). For example, sympathetic nerves are rich in human intrapulmonary arteries, but absent in rat intrapulmonary arteries (reviewed by Barnes and Liu, 1995). The stimulation of sympathetic nerves of the pulmonary circulation increases pulmonary vascular resistance and pulmonary arterial pressure, which can be blocked by an α-adrenoceptor antagonist and abolished by chemical sympathectomy using 6-hydroxydopamine (Liu & Barnes, 1994). The stimulation of parasympathetic nerves induces pulmonary vasodilation via muscarinic receptors on pulmonary vascular endothelium leading to NO release (Liu & Barnes, 1994).

In the periphery, catecholamines, NA and adrenaline (Adr) are stored and released from two primary sites. Post-ganglionic sympathetic neurons are the major source of NA, whereas Adr is predominantly released along with NA into the blood stream from the adrenal medulla (O'Rourke & Vanhoutte, 1992). α_2-Adrenoceptors and β_2-adrenoceptors located on sympathetic nerve endings modulate the release of NA from neurons. The activation of pre-junctional β_2-adrenoceptors and α_2-adrenoceptors cause the increase and inhibition of NA release from the sympathetic nerve system, respectively (Vanhoutte *et al.*, 1981).

NA and Adr can activate α-adrenoceptors (α_1- and α_2-subtypes) inducing vasoconstriction and β-adrenoceptors (β_1-, β_2- and β_3-subtypes) inducing vasodilation in pulmonary vasculature (O'Rourke & Vanhoutte, 1992; Salvi, 1999). However, α_1-adrenoceptors are the primary receptors, through which sympathetic nerves act in the pulmonary circulation (Barnes & Liu, 1995). The α_1-adreneceptors that are present in the small- and medium-sized pulmonary arteries mediate not only smooth muscle contraction but also proliferation and growth in humans (Salvi, 1999).

Sympathetic nerves may play a role in the maintenance of basal pulmonary vascular tone; however the pulmonary circulation receives less influence from parasympathetic innervation (Fishman, 1976; Barnes & Liu, 1995). Much evidence shows that hypoxia apparently stimulates the sympathetic nervous system, including local synthesis, storage or release of catecholamines (Fishman, 1976; Heistad & Abboud, 1980). The chemoreceptors are also involved in the regulation of pulmonary vascular tone (Bamford *et al.*, 1999; Prabhakar, 2000). The carotid body chemoreceptors which are located at the bifurcation of the common carotid artery are sensitive to oxygen tension by its type I cells (Brij & Peacock, 1998). Hypoxia stimulates type I cells to release the transmitter dopamine, which activates the afferent fibers of the carotid sinus nerve and activates the sympathetic nervous system. This results in vasoconstriction in muscle, redistribution of blood flow, increase of cardiac output, pulmonary vasoconstriction and hyperventilation (Lopez-Lopez *et al.*, 1989; Sugito *et al.*, 1998).

Sympathetic motor innervation is not necessary for the pulmonary pressor responses to hypoxia (Von Euler & Liljestrand, 1946; Reeves & Rubin, 1998). This conclusion is based on the fact that HPV has been demonstrated in various experimental models *in vivo* and *in vitro*. It is observed in pulmonary artery rings and in single pulmonary artery smooth muscle cells (Bennie *et al.*, 1991; Madden *et al.*, 1992; Weir & Archer, 1995; Liu *et al.*, 2001). Also, the isolated lung preparation in which hypoxia can produce HPV is thought to be devoid of sympathetic connections (Fishman, 1976). However, this does not exclude a modulatory role for circulating catecholamines in hypoxic pulmonary hypertension. Prolonged exposure to hypoxia increases the levels of circulating NA and Adr (reviewed by Rostrup, 1998). This, coupled with the fact that hypoxia up-regulates α1-adrenoreceptor density and down-regulates β-adrenoreceptor density in pulmonary arteries, would shift the balance further in favour of vasoconstriction and proliferative responses (Xie *et al.*, 1991).

The pulmonary circulation is also an important site for clearance of circulating catecholamines in various species, including human, rat, rabbit, and dog lungs, mainly by uptake and metabolism in endothelial cells of the lung microvasculature (reviewed by Bakhle and Vane, 1974). There are two distinct mechanisms involved in catecholamine uptake, $Uptake_1$ and $Uptake_2$, corresponding to neuronal and extra-neuronal uptake, respectively. They have different kinetic properties as well as different substrates and inhibitor specificities. $Uptake_1$ is a high-affinity system with a relatively low maximum rate of uptake, whereas $Uptake_2$ has low affinity for NA, but a much higher maximum rate (Iversen *et al.*, 1971; Bakhle & Vane, 1974). The substrate specificity is also

different for $Uptake_1$ and $Uptake_2$; $Uptake_1$ is relatively selective for NA, whereas $Uptake_2$ also accumulates Adr and isoprenaline. After uptake, catecholamines are metabolised by monoamine oxidase and catechol-O-methyl transferase.

1.6 Mediators involved in hypoxic pulmonary hypertension

It is presumed that a balance between the effects of endogenous vasodilators and vasoconstrictors regulates pulmonary vascular tone (MacLean, 1999a). Hypoxic pulmonary hypertension could result from either an increase of vasoconstrictor activity, e.g. ET-1, catecholamines, Ang II, 5-HT and thromboxane A_2 (TxA_2), or a decrease of vasodilator, e.g. nitric oxide (NO), prostacyclin (PGI_2) influences.

1.6.1 ET-1

ET-1 is a 21-amino acid peptide, which was first isolated from the supernatant fraction of cultured endothelial cells and found to have potent vasoactive properties by Yanagisawa *et al.* (1988). Endothelins (ETs) represent a family of structurally-related peptides, which have three ET isoforms (ET-1, ET-2 and ET-3). Human ET-1 is derived from a 212-amino acid precursor, prepro-endothelin, via a 38-amino acid intermediate, big ET-1. An endothelin-converting enzyme cuts big ET-1 to mature ET-1. Conversion to ET-1 amplifies big ET-1 activity at least 100-fold (Hisaki *et al.*, 1994). Obviously, the conversion of big ET-1 to ET-1 is a crucial step in the formation of the biologically active peptide. Many factors like hypoxia, thrombin, transforming growth factor beta and shear stress can induce ET production (Kourembanas *et al.*, 1991; Wort *et al.*, 2001). Phosphoramidon, a metalloproteinase inhibitor, markedly suppresses the conversion process of big ET-1 into the mature form (Hisaki *et al.*, 1994; Xu *et al.*, 1994).

Two types of ET receptors have been cloned to date, named as ET_A and ET_B receptors. The two classes of ET receptors are distinct in their ligand binding affinity and distribution in tissues. ET_A and ET_B receptors co-exist on the pulmonary vascular smooth muscle (Lal *et al.*, 1996). ET_A receptors have stronger affinity for ET-1; the activation of ET_A receptors mediates smooth muscle contraction and cell proliferation (Wort *et al.*, 2001). ET_B receptors have equal affinity towards all three isoforms of ET; the activation of ET_B receptors causes vasoconstriction through the ET_{B2} sub-type and vasodilatation through the ET_{B1} sub-type in the pulmonary circulation. ET_{B2} receptors are located on the pulmonary vascular smooth muscle cells. ET_{B1} receptors, which mediate vasodilation by releasing vasodilators PGI_2 and NO, are located on the pulmonary endothelial cells, (MacLean *et al.*, 1994b; Lal *et al.*, 1996). Lower concentrations of ET_B receptor agonist elicit pulmonary vasodilation via ET_{B1} receptors, whereas higher concentrations cause pulmonary vasoconstriction via ET_{B2} receptors. There are regional differences in the distribution of ET receptors within pulmonary vasculature. In extrapulmonary artery rings, ET-1 induces contractile responses via ET_A receptors, whereas in intrapulmonary resistance arteries, ET-1 induces vasoconstriction mainly via ET_B receptors (LaDouceur *et al.*, 1993; MacLean *et al.*, 1994b). In the whole lung, ET-1 operates mainly through the ET_A receptors in the pulmonary vasculature. BQ-123, a selective ET_A receptor antagonist attenuated the ET-1-induced vasoconstriction responses in isolated perfused rat lungs, whereas BQ788, a selective ET_B receptor antagonist potentiated the vasoconstriction responses to ET-1 (Uhlig & Featherstone, 1997). The lung is also a major site for the clearance of ET-1 and ET_B receptors contribute to the clearance of ET-1 (Dupuis *et al.*, 1996).

ETs are likely to contribute to the pathogenesis of hypoxic pulmonary hypertension because of their ability to cause vasoconstriction and act as co-mitogens for vascular smooth muscle and pulmonary arterial fibroblasts (reviewed by Michael & Markewitz, 1996). It has been shown that ET-1 concentration was elevated in the cultured endothelial cells exposed to hypoxia (Kourembanas *et al.*, 1991). The plasma concentration of ET-1 was elevated in rats with hypoxic pulmonary hypertension and in patients with pulmonary hypertension (Stewart *et al.*, 1991; Yoshibayashi *et al.*, 1991). Furthermore ET converting enzyme activity is up-regulated in CH lungs (Li *et al.*, 1999; Lal *et al.*, 2000). Much evidence has shown that ET-1 contributes to hypoxic pulmonary hypertension. BQ123 abolished HPV in porcine distal pulmonary arteries (Liu *et al.*, 2001). Exposure to hypoxia increased the number of ET_A receptors in the media of pulmonary resistance arteries (Jones & Morice, 1998). BQ-123 completely prevented the development of pulmonary artery hypertension and vascular remodelling in rats during CH (Chen *et al.*, 1993; DiCarlo *et al.*, 1995). Bosentan, a combined ET_A and ET_B receptor antagonist, can protect rats against the development of pulmonary hypertension and right ventricular hypertrophy during CH (Eddahibi *et al.*, 1995). Therefore, ET-1 might be a very important mediator for hypoxic pulmonary hypertension.

1.6.2 Ang II

Renin is released into the circulation from the kidney and converts angiotensinogen into angiotensin I in the plasma. Angiotensin I is converted by angiotensin converting enzyme

into Ang II, mainly in the endothelium of pulmonary vasculature (Ng & Vane, 1967; Ito *et al.*, 1988). Ang II effects are mediated via AT_1 and AT_2 receptors. AT_1 are widely distributed throughout adult tissues, whereas AT_2 are abundantly expressed throughout fetal tissues but decrease dramatically and rapidly after birth (Csikos *et al.*, 1998).

Ang II is a potent agonist that causes contraction of vascular smooth muscle via the activation of AT_1 receptors (Brailoiu *et al.*, 1999; Kotani *et al.*, 1999). Ang II is also a mitogenic factor, which induces proliferation of lung fibroblasts and vascular smooth muscle cells demonstrated by *in vitro* studies (Marshall *et al.*, 2000). This mitogenic effect of Ang II is related to the stimulation of transforming growth factor β (Koibuchi *et al.*, 1993; Siegert *et al.*, 1999). In addition, Ang II can stimulate collagen production in vascular smooth muscle cells, demonstrated in human arterial smooth muscle cells (Ford *et al.*, 1999).

It has been shown that angiotensin converting enzyme activity is enhanced in the small muscular pulmonary arteries during CH (Morrell *et al.*, 1995a). Furthermore, angiotensin converting enzyme inhibitors, for example perindopril, captopril or quinapril can reduce the pulmonary wall thickness in CH rats (Morrell *et al.*, 1995a ; Jeffery & Wanstall, 1999). Supporting evidence comes from experiments showing that less pulmonary vascular remodelling appeared in angiotensin converting enzyme-deficient mice compared to wild-type mice during CH (Suylen *et al.*, 2001). Thus, Ang II may contribute to pulmonary vascular remodelling during hypoxia and AT_1 receptors are responsible for the effect of endogenous Ang II in the pulmonary vascular remodelling

induced by hypoxia (Morrell *et al.*, 1995b).

1.6.3 5-HT

5-HT is produced mainly in the enterochromaffin cells of the intestine, and is also released from pulmonary neuroendocrine cells and neuroepethelial bodies distributed throughout the airway (MacLean *et al.*, 2000). Circulating 5-HT is stored in the platelets; so free 5-HT in plasma is extremely low. 5-HT is mainly taken up by the lung and inactivated by the pulmonary endothelial cells (Vane, 1969).

5-HT has vasoconstrictor and mitogenic effects in the pulmonary circulation (Eddahibi *et al.*, 2000). 5-HT receptor sub-types, $5HT_{1D/1B}$ and $5\text{-}HT_{2A}$ are involved in vasoconstrictor responses in pulmonary arteries of humans and rats (Cortijo *et al.*, 1997; Shaw *et al.*, 2000).

One important finding linking 5-HT and pulmonary hypertension is that patients taking anorexigens (e.g. fenfluramine) can develop pulmonary hypertension. Such drugs increase 5-HT release from the platelets; inhibit 5-HT uptake and monoamine oxidase activity that metabolizes 5-HT (MacLean, 1999a). Fawn-hooded rats also demonstrate the relationship between 5-HT and pulmonary hypertension. Pulmonary hypertension develops in fawn-hooded rats, which is thought to be due to 5-HT platelet storage deficiency (Gonzalez *et al.*, 1998). When knock-out of the 5-HT transporter gene was undertaken, the mice developed less pulmonary hypertension and pulmonary vascular

remodelling during CH compared to the controls (Eddahibi *et al.*, 2000). Therefore, 5-HT is also implicated in hypoxic pulmonary hypertension.

1.6.4 TxA_2

The formation of TxA_2 from prostaglandin H_2 is catalyzed by TxA_2 synthase. TxA_2 has been shown to possess a variety of biological effects, the most important effects of which are platelet aggregation and constriction of vascular smooth muscle (Raj *et al.*, 1992; Bauer *et al.*, 1999). TxA_2 can induce vasoconstriction in pulmonary arteries. And also it has been demonstrated that TxA_2 is present in the pulmonary vascular smooth muscle cells in rat intrapulmonary arteries (Ermert *et al.*, 2000). Increased production of TxA_2 has been found in patients with primary pulmonary hypertension or chronic obstructive pulmonary disease by measuring the metabolite of TxA_2 in urine (Christman *et al.*, 1992; Davi *et al.*, 1997). However, the role of TxA_2 in hypoxic pulmonary hypertension needs to be extensively investigated.

1.6.5 Vasodilators and hypoxic pulmonary hypertension

During hypoxia, the increase of pulmonary vascular resistance could be due to a decrease of vasodilator influence. As a potent vasodilator, NO could contribute to the regulation of pulmonary vascular tone. However, most studies have shown that hypoxia increases NO production, even though some groups have shown that hypoxia inhibits NO synthesis in the pulmonary artery (Johns *et al.*, 1989; Fike *et al.*, 1998; Berkenbosch *et al.*, 2000;

Quinlan *et al.*, 2000). NO release was enhanced in the pulmonary circulation during acute hypoxia because N-monomethyl-L-arginine (L-NMMA), an inhibitor of NO synthase (NOS), increased HPV in *in vivo* and *in vitro* studies (Robertson *et al.*, 1990; Liu *et al.*, 1991; Sprague *et al.*, 1992; Liu *et al.*, 2001). Similar to acute hypoxia, during CH, L-NMMA and L-NAME also enhanced pulmonary arterial pressure in the isolated lungs of rats (Barer *et al.*, 1993). CH induced up-regulation of the endothelial isoform of NOS (eNOS) in the endothelium of both large and small pulmonary arteries (Xue *et al.*, 1994; Hampl *et al.*, 1995; Le Cras *et al.*, 1996; Tyler *et al.*, 1999). Northern blot and immunohistochemical analyses also showed that the inducible isoform of NOS (iNOS) was enhanced in the vascular smooth muscle of large and small pulmonary arteries from CH rats (Xue & Johns, 1996). Furthermore, immunohistochemistry has demonstrated that the expression of soluble guanylyl cyclase, the primary receptor for NO, is increased in the smooth muscle cells of small pulmonary arteries and arterioles in hypoxic rat lungs (Li *et al.*, 1999). Much severer pulmonary hypertension and pulmonary remodelling was seen in eNOS-knockout mice during CH than normal or eNOS-knockout mice in normoxia (Steudel *et al.*, 1998; Fagan *et al.*, 1999). Thus, a decrease in activity of the NO system is not the cause of hypoxic pulmonary hypertension during hypoxia. In fact the increases reported may serve to counterbalance the increase of pulmonary vasoconstriction induced by hypoxic stress.

PGI_2 is another vasodilator, which is released from the pulmonary endothelial cells (Tuder *et al.*, 1999). During acute hypoxia, the cyclo-oxygenase inhibitor indomethacin which blocks the initial step in the formation of prostaglandins (Ermert *et al.*, 1998) had

no effect on HPV induced in porcine distal pulmonary arteries (Liu *et al.*, 2001). The expression of PGI_2 synthase in the pulmonary endothelium detected by immunohistochemistry analysis was decreased in the small and medium-sized pulmonary arteries from patients with primary pulmonary hypertension compared to normal pulmonary arteries (Tuder *et al.*, 1999). Similarly, the production of PGI_2 detected in the endothelium was decreased in the conductive and resistance pulmonary artery rings after CH exposure (Badesch *et al.*, 1989). Also, a recent study has shown that pulmonary hypertension, pulmonary vascular remodelling and right ventricular hypertrophy after CH were developed more severely in PGI_2 receptor-knockout mice compared to wild-type mice (Hoshikawa *et al.*, 2001). It seems that a deficit of PGI_2 production in the pulmonary endothelium during CH could be one factor contributing to hypoxic pulmonary hypertension.

1.7 Signalling pathways in vascular smooth muscle contraction

Vasoconstrictors such as Ang II, ET-1 and α-adrenoceptor agonists bind to their specific receptors on vascular smooth muscle cells and induce vessel contraction. Although the receptors are different, they are all guanine nucleotide-binding protein (G protein)-coupled receptors. After agonists bind to G protein-coupled receptors, this binding hydrolyses phosphatidylinositol 4,5-bisphosphate by a phosphoinositide-specific phospholipase to generate two second messengers, inositol 1,4,5-triphosphate (IP_3) and 1,2-diacyl-sn-gylcerol (DAG) (Karaki, 1989; Hamada *et al.*, 1997; Bauer *et al.*, 1999). DAG can activate PKC, which has multiple functions in smooth muscle cells.

IP_3 enters the cytoplasmic compartment whereby it has an agonist effect at specific IP_3 receptors located on sarcoplasmic reticulum (SR) in the smooth muscle cells. The activation of these IP_3 receptors causes release of Ca^{2+} from the intracellular stores. Ca^{2+} released from the IP_3-sensitive stores has both positive and negative feedback on its own release through altering the affinity of the receptor. The emptying of Ca^{2+} from IP_3-sensitive Ca^{2+} stores has been proposed to promote Ca^{2+} influx through voltage-gated Ca^{2+} channels, thereby sustaining contraction (Gelband & Gelband, 1997). So, both Ca^{2+} release and Ca^{2+} influx are dependent on the formation of IP_3 (Hamada *et al.*, 1997; Hu *et al.*, 1999). However, Ca^{2+} influx induced by membrane depolarization is the major source of $[Ca^{2+}]_i$ increase induced by agonists (reviewed by McFadzean & Gibson, 2002).

Not all Ca^{2+} stores in the SR are mediated by IP_3 receptors, another intracellular Ca^{2+}-

release channel is IP_3 receptor-insensitive but sensitive to Ca^{2+} and caffeine and can be inhibited by ryanodine, the so-called 'ryanodine receptor' (Ito *et al.*, 1986; Sorrentino & Reggiani, 1999). Ryanodine receptors mainly exist in skeletal and cardiac muscles. Ca^{2+} acts on the ryanodine receptors on the SR, inducing Ca^{2+} release, known as Ca^{2+}-induced Ca^{2+} release (CICR) (Itoh *et al.*, 1992; Maxwell *et al.*, 1998). So, membrane depolarization of vascular smooth muscle cells, induced by vasoconstrictors or high K^+, opens L-type Ca^{2+} channels on the membrane leading to Ca^{2+} influx (Karaki *et al.*, 1997); Ca^{2+} influx triggers an explosive release of stored Ca^{2+} from the SR (Neylon *et al.*, 1995). However, there is no evidence showing that Ca^{2+} influx is able to activate Ca^{2+} release via IP_3 receptors.

Following receptor activation induced by agonists on the membrane of the smooth muscle cells, increased free Ca^{2+} in the cells is returned to resting levels by uptake into SR via Ca^{2+}-ATPase and removal from the cells via Na^+-Ca^{2+} exchangers on the plasma membrane. Thapsigargin, a Ca^{2+}-ATPase inhibitor blocks Ca^{2+}-ATPase on the SR and thus can deplete the Ca^{2+} store in SR (Maxwell *et al.*, 1998; Ivery *et al.*, 1999; Shimoda *et al.*, 2000). Mitochondria are also involved in Ca^{2+} mobilization in vascular smooth muscle cells (Drummond & Tuft, 1999; Lagaud *et al.*, 1999; Doi *et al.*, 2000a).

1.8 The mechanism of vascular smooth muscle contraction-----sliding theory

The contractile apparatus of vascular smooth muscle includes thick and thin filaments, mainly comprising myosins and actins, respectively (Chien, 1990). Vascular myosin is composed of two heavy chains and two sets of light chain units (regulatory and alkali light chains) (Hathaway *et al.*, 1991). The 'heads' (cross-bridges) of myosins, which are bundled together into thick filaments, undergo a cycle of high- and low-affinity binding to actins, energized by ATP hydrolysis (Walker *et al.*, 1994).

A change of $[Ca^{2+}]_i$ is the main mechanism which initiates vascular smooth muscle contraction and relaxation. The resultant increase in $[Ca^{2+}]_i$ mediated by vasoconstrictors enhances the binding of Ca^{2+} to calmodulin (CaM). A Ca^{2+}-CaM complex activates myosin light chain kinase to phosphorylate the regulatory myosin light chain. This simple phosphorylation reaction triggers cycling of myosin cross-bridges along actin filaments, resulting in the sliding of the thick filaments and the thin filaments past each other and smooth muscle contraction. Following a reduction in $[Ca^{2+}]_i$ the regulatory myosin light chain is dephosphorylated, resulting in smooth muscle relaxation (Karaki *et al.*, 1997).

Obviously regulatory myosin light chain phosphorylation is the pivotal factor that determines the extent of smooth muscle contraction. A balance between the activity of the Ca^{2+}-dependent myosin light chain kinase and the Ca^{2+}-independent myosin light chain phosphatase determines the extent of phosphorylation of myosin light chain. Myosin light chain kinase phosphorylates regulatory myosin light chain whereas myosin

phosphatase catalyzes dephosphorylation of regulatory myosin light chain (Walsh, 1991). In other words, a decrease of the activity of myosin light chain phosphatase can enhance regulatory myosin light chain phosphorylation and induce smooth muscle contraction.

Differing from cardiac and skeletal muscles, vascular smooth muscle often remains in a contracted state for a long period while regulatory myosin light chain phosphorylation and $[Ca^{2+}]_i$ decrease. This mechanical state of tension maintenance with slowly cycling of cross-bridges has been called the 'Latch State' (Hai & Murphy, 1989). $[Ca^{2+}]_i$ during the latch state is much lower than in the early stage of smooth muscle contraction although Ca^{2+} is necessary for maintenance of the latch state. This latch state could be due to slowing of cross-bridge detachment (Hathaway *et al.*, 1991). However, there are no specific reports that changes of the latch state accounted with pulmonary vascular hyper-reactivity during CH.

1.9 The role of Ca^{2+} sensitization in vascular smooth muscle contraction

Not only does $[Ca^{2+}]_i$ play an important role in determining contractions of vascular smooth muscle, but also Ca^{2+} sensitization of smooth muscle myofilaments provides a key determinant of contractile force (Karaki, 1989). Ca^{2+} sensitization can be defined as the degree of vasoconstrictor response elicited above that expected for a given $[Ca^{2+}]_i$. So, myosin light chains get phosphorylated through a Ca^{2+} independent pathway, which induces smooth muscle cell contraction.

The existence of Ca^{2+} sensitization was demonstrated in experiments using permeabilized vessels. Agonists can increase force of contraction in permeabilized smooth muscle in which the $[Ca^{2+}]_i$ is clamped with Ca^{2+} chelators and intracellular stores are depleted (Nishimura *et al.*, 1992; Evans *et al.*, 1999).

Inhibition of myosin light chain phosphatase could be a major mechanism of Ca^{2+} sensitization (Feng *et al.*, 1999; Robertson *et al.*, 2000; Fukata *et al.*, 2001). Rho, which activates Rho-kinase and as a result, suppresses myosin light chain phosphatase activity, is demonstrated to be involved in agonist-induced Ca^{2+} sensitization in smooth muscle cells (Fukata, *et al.*, 2001).

PKC might be also involved in Ca^{2+} sensitization in smooth muscle cells. In aortic rings, NA-induced contraction was potentiated by ET-1 in the absence of changes in stimulated Ca^{2+} entry, and staurosporine and calphostin, PKC inhibitors, prevented the potentiation

effect of ET-1 (Henrion & Laher, 1993). It is thought that PKC sensitizes smooth muscle cells via inhibiting myosin light chain phosphatase (Nishimura *et al.*, 1989; Weissman *et al.*, 1999).

It has been demonstrated that tyrosine kinase plays a role in Ca^{2+} sensitization in the mesenteric artery, but not in the pulmonary artery as Tyrphostin A23, a selective tyrosine kinase inhibitor, inhibited ET-1-induced vasoconstriction in permeabilized mesenteric arteries but not in permeabilized pulmonary arteries (Ohanian, *et al.*, 1997; Evans, *et al.* 1999).

Intracellular pH (pHi) is another important determinant for Ca^{2+} sensitization in vascular smooth muscle. pHi can vary due to changes of extracellular pH or the action of agonists and changes in pHi may affect $[Ca^{2+}]_i$ or affect the affinity of CaM with Ca^{2+} (Smith *et al.*, 1998). Intracellular alkalinization causes increases of $[Ca^{2+}]_i$ and increases of pulmonary arterial pressure, as demonstrated in isolated ferret lungs. Furthermore, maintaining intracellular alkalinization causes a further increase of pulmonary arterial pressure without any change of $[Ca^{2+}]_i$ (Farrukh *et al.*, 1996).

The Na^+/H^+ exchanger and $Na^+/HCO3^-$ exchanger play a crucial role in the regulation of pHi as demonstrated in cultured guinea pig or ferret pulmonary arterial smooth muscle cells (Quinn *et al.*, 1991; Farrukh *et al.*, 1996). The Na^+/H^+ exchanger extrudes protons from the smooth muscle cells. So, inhibition of the Na^+/H^+ exchanger decreases pHi (Farrukh *et al.*, 1996). Agonist activation of vascular smooth muscle (e.g. ET-1) may

involve increases in the activity of the Na^+/H^+ and the $Na^+/HCO3^-$ transporters and result in intracellular alkalinization and indirectly increase Ca^{2+} sensitization (Wang *et al.*, 2000).

1.10 Aims of this study

Pulmonary vascular hyper-reactivity during CH has not been investigated extensively and its mechanism is unknown. Previous studies have shown that ET-1 is involved in HPV and pulmonary vascular remodelling during CH because of its ability to cause vasoconstriction and act as co-mitogens for vascular smooth muscle and pulmonary arterial fibroblasts (reviewed by Michael & Markewitz, 1996). ET-1 can also sensitize the myofilaments to Ca^{2+} in pulmonary vascular smooth muscle cells (Evans *et al.*, 1999). Therefore, ET-1 might play a role in the pulmonary vascular hyper-reactivity during CH. Emery et al. (1981) think that the potentiation of vasoconstrictor responses may be due to the pulmonary arterial remodelling. Because this new muscle encroaches on the vascular lumen and makes it narrower, a given degree of muscle shortening, which reduces the vessel circumference, will cause a larger percentage reduction in radius and a higher increase in vascular pressure. The relationship of pulmonary arterial muscularization and pulmonary vasoconstrictor responses during CH is under investigation to assess the role of pulmonary vascular remodelling in the pulmonary vascular hyper-reactivity.

Hence, the aims of this study are:

1) To investigate the effect of CH on the vasoconstrictor responses to agonists in isolated perfused rat lungs and pulmonary artery rings.

2) To determine whether endogenous ET-1 production mediates pulmonary vascular hyper-reactivity in the isolated perfused lungs from CH rats.

3) To investigate the effects of ET-1 on vasoconstrictor responses to other agonists in normoxic isolated perfused lungs and the signalling pathways involved.

4) To determine the relationship between pulmonary vascular remodelling and pulmonary vascular hyper-reactivity during CH.

5) To determine whether effects of CH on catecholamine uptake can account for pulmonary vascular hyper-reactivity in isolated perfused lungs.

CHAPTER TWO

METHODOLOGY

2.1 Hypoxic animal model

For CH animals, male Wistar rats (240 - 260g) were maintained in a chamber of 10% O_2 (v/v) for 3 weeks. The chamber was kept at room temperature (22 - 23°C) and suitable humidity (50 - 70%) in a re-circulating normobaric environment (Lal *et al.*, 1999a). The re-circulating air was passed through a silica gel column to remove water vapour, while CO_2 was absorbed through a soda lime column. Oxygen tension was continuously monitored using a Servomex 1440C gas analyzer (Lal *et al.*, 1999a). Nitrogen was pumped into the chamber when O_2 tension was more than 10.5%; and air was pumped into the chamber when O_2 tension was less than 9.5%. The animals were used for experiment within 1 hour of removal from the chamber. For recovery studies, rats were transferred to room air after 3 weeks in the hypoxic chamber and utilized up to 3 weeks later. As indices of hypoxic pulmonary hypertension, haematocrit and ratio of right ventricular weight/total heart weight were measured. Only the free wall of the right ventricle, not including septum was weighed as right ventricular weight.

2.2 Isolated perfused lung preparation

Male Wistar rats were terminally anaesthetised with pentobarbital sodium (100mg/kg) intraperitoneally and heparinized (500IU) via the tail vein. The lungs were isolated after the pulmonary artery was cannulated via the right ventricle and the trachea was cannulated. The right and left ventricles were cut off to allow free efflux of fluid. The isolated lungs were perfused with Krebs' solution from the cannula in the pulmonary

artery, aerated by 20% O_2 / 75% N_2 / 5% CO_2, pH 7.3. The perfusion flow was fixed at 5ml/min, 37 °C .The lungs were ventilated with room air, at 28 strokes/min. Pulmonary perfusion pressure (PPP) and pulmonary inflation pressure (PIP) were recorded through pressure transducers, which connected to the pulmonary artery cannula and the tracheal cannula, respectively. The effluent perfusate passed through left atrium and was not recirculated. Lungs were suspended from an isometric transducer for recording changes of lung weight (Figure 2.1, part A) (Lal *et al.*, 1994).

Lungs were equilibrated for 15 min before adding drugs. Agonists were bolus-injected or infused into the pulmonary artery. Antagonists were infused for 15 min before injection of agonists.

For the study of ET-1 sensitization, ET-1 was infused for 30 min in normoxic lungs before bolus-injections of PHE, Ang II and KCl. For antagonist-treated groups, antagonists were infused during the last 15 min of ET-1 infusion.

In a series of experiments, the isolated lungs were perfused with low oxygen, 10% O_2 / 85% N_2 / 5% CO_2, pH at 6.56.

2.3 Rat stomach strip preparation for bioassay

For study of catecholamine metabolism in the lung, a rat stomach strip (RSS) was prepared from the fundus of rat stomach (Armitage and Vane, 1964), cut along the

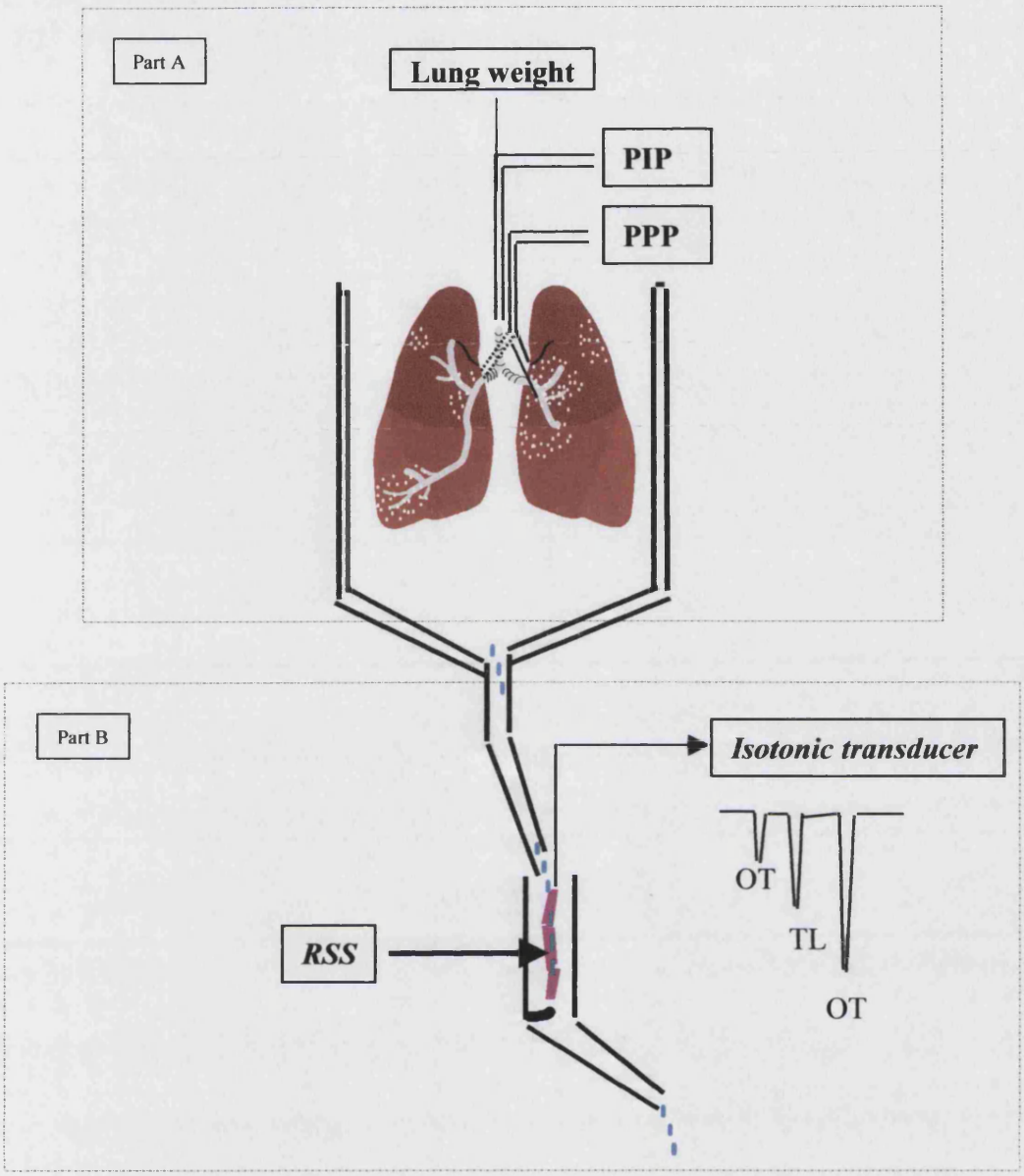

Figure 2.1: Schematic illustration of isolated perfused lung and rat stomach strip preparation

Part A: Isolated perfused lung preparation. The lung was suspended and connected to an isometric transducer recording changes of lung weight. Trachea and pulmonary artery were cannulated and connected to pressure transducers, recording PPP and PIP respectively.

Part B: RSS preparation. Infusions of NA or Adr were over the RSS tissue (OT) directly or through the lung (TL). The changes of length of the RSS were recorded via an isotonic transducer.

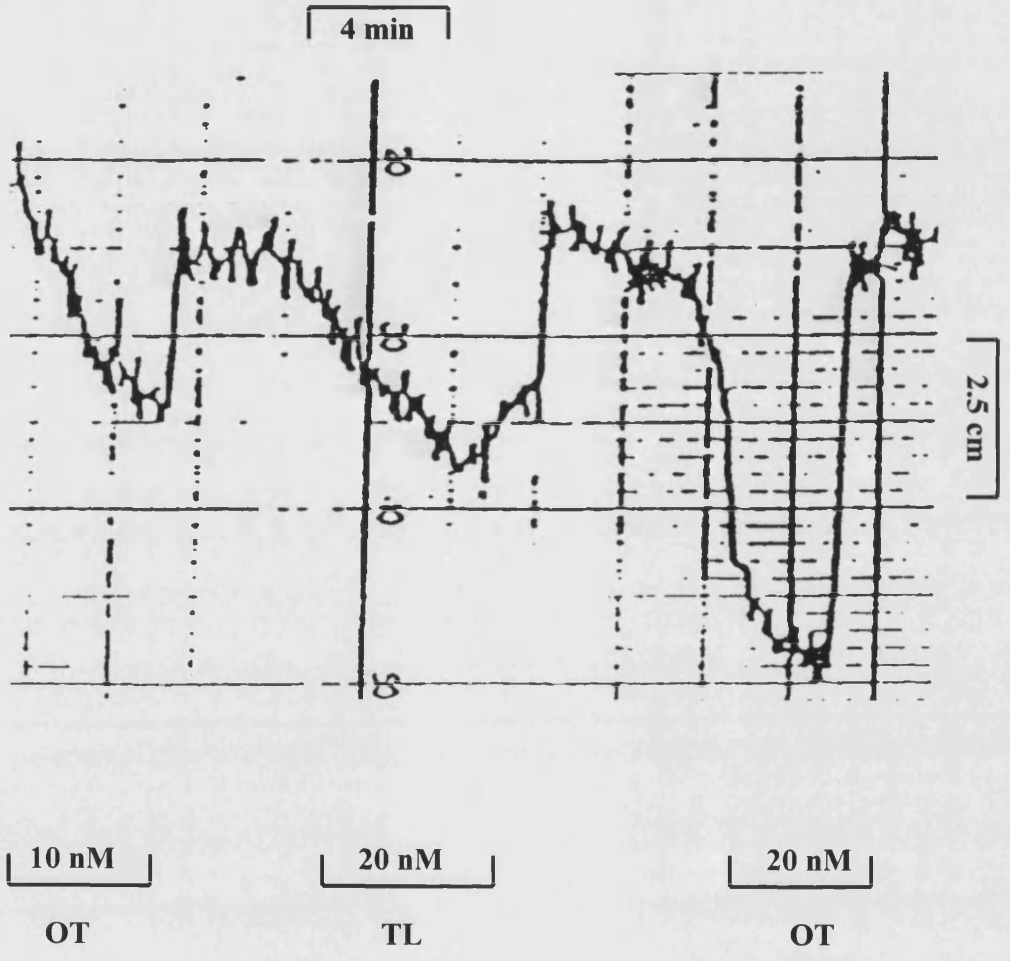

Figure 2.2: A typical experimental trace showing removal of NA in the isolated perfused lung.

The relaxation of the rat stomach strip was induced by NA, which was infused over the tissue directly (OT) or infused through the lung (TL). The rat stomach strip was pre-contracted with 5-HT.

longitude, about 3 - 4 cm long and 1.5 - 2mm wide. The RSS was set up in an organ bath and superfused with Krebs' solution at 5ml/min and 34°C. The upper end of the tissue was attached to an isotonic lever to record the change of tension. The load on the lever was 1 g.

5-HT (1.5 - 3 μM) was infused over the stomach strip for pre-contraction (Armitage & Vane, 1964). The stomach strip relaxed when NA (5 nM - 20 nM) or Adr (1 nM - 15 nM) was infused (5 min) through the lung or over the RSS tissue directly. The removal of catecholamines by lungs was determined by bracketing the RSS responses to catecholamines superfused over the RSS directly and through the lungs (Figure 2.1, part B and Figure 2.2) (Alabaster & Bakhle, 1973).

2.4 Isolated aortic ring preparation

Aortic rings (3 mm long) were dissected gently from thoracic aortae in normoxic or CH rats. The vessels were set up in 10ml organ baths with Krebs' solution, bubbled with 95% O_2/5% CO_2 at 37 °C. The aortic rings were equilibrated for 1 hour before starting experiments. Resting tension was set at 1g and contraction of rings was recorded via an isometric transducer. Cumulative concentration-responses to PHE were recorded by a Maclab system.

2.5 Isolated perfused mesenteric bed

The superior mesenteric artery was cannulated via the aorta and the colic artery was tied off. The mesenteric vascular bed was separated from the intestine and aorta and perfused with Krebs' solution aerated by 95% O_2/5% CO_2, pH 7.3. The perfusion flow was 5ml/min, 37°C. Mesenteric perfusion pressure was recorded via a pressure transducer. All preparations were allowed to equilibrate for 15 min before drug administration.

2.6 Histologic analysis of pulmonary vasculature

Rats were anaesthetised with pentobarbital sodium (100mg/kg i.p.) and heparinized (500IU i.v.). The pulmonary artery was cannulated via the right ventricle. The heart was cut off to allow free efflux of perfusate. The isolated lungs were perfused with Krebs' solution from the cannula in the pulmonary artery at a constant flow rate (5ml/min) for 5 min, 37°C, then changed from Krebs' solution to formol saline (0.4%) and perfused with formal saline for 5 min. The lungs (n = 5 rats / each group) were immersed in formol saline for at least 1 week. Three different lobes from the right lungs were embedded in paraffin separately. Transverse slices (4μm thickness) of lung tissue were taken from the middle of each lobe and stained with haematoxylin and eosin.

Sections were viewed under a microscope (JVC, KY-F55BF, Japan) with a computer analysis system (ZEISS, KS300). Measurements were made of external arterial diameter and medial thickness at 100 ~ 400 magnification. Pulmonary arteries were identified by accompanying airway and pulmonary veins (Figure 2.3). Histological changes were quantified as described by Hislop and Reid (1976). Vessel diameter was defined as the

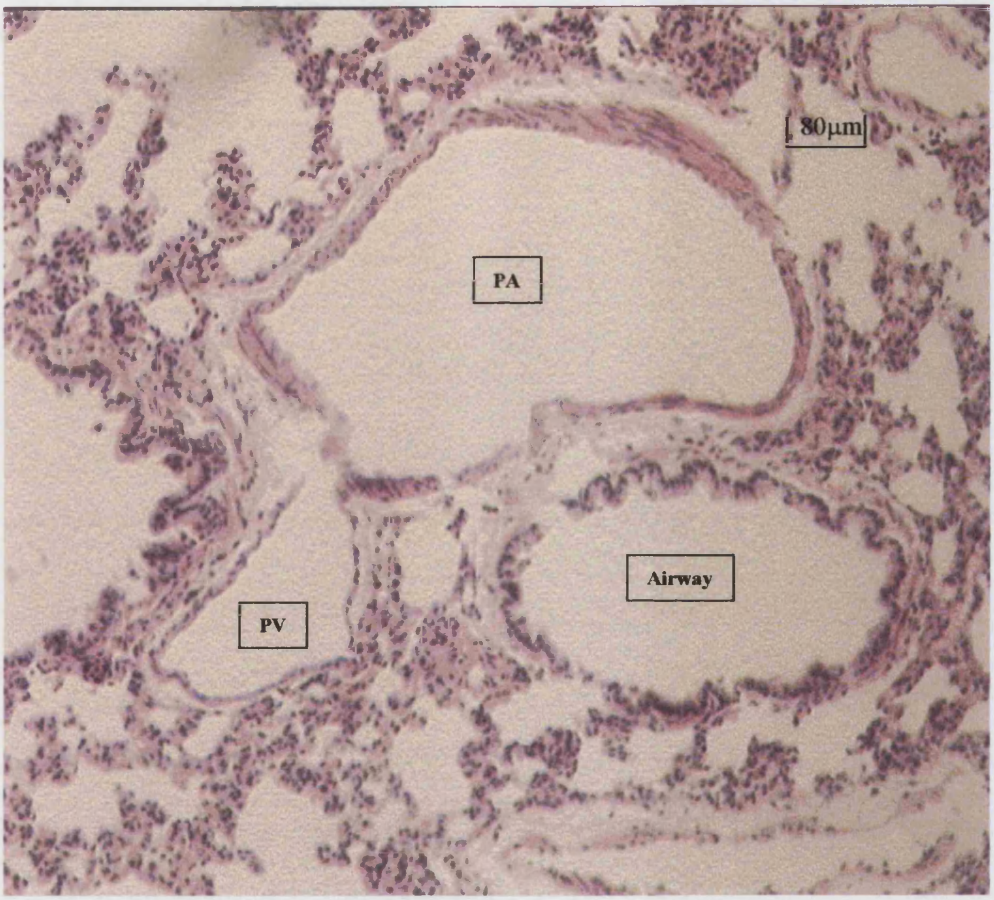

Figure 2.3: Representative micrograph showing the location of pulmonary artery

Pulmonary artery (PA) was identified by accompanying airway and pulmonary vein (PV), ×100 magnification.

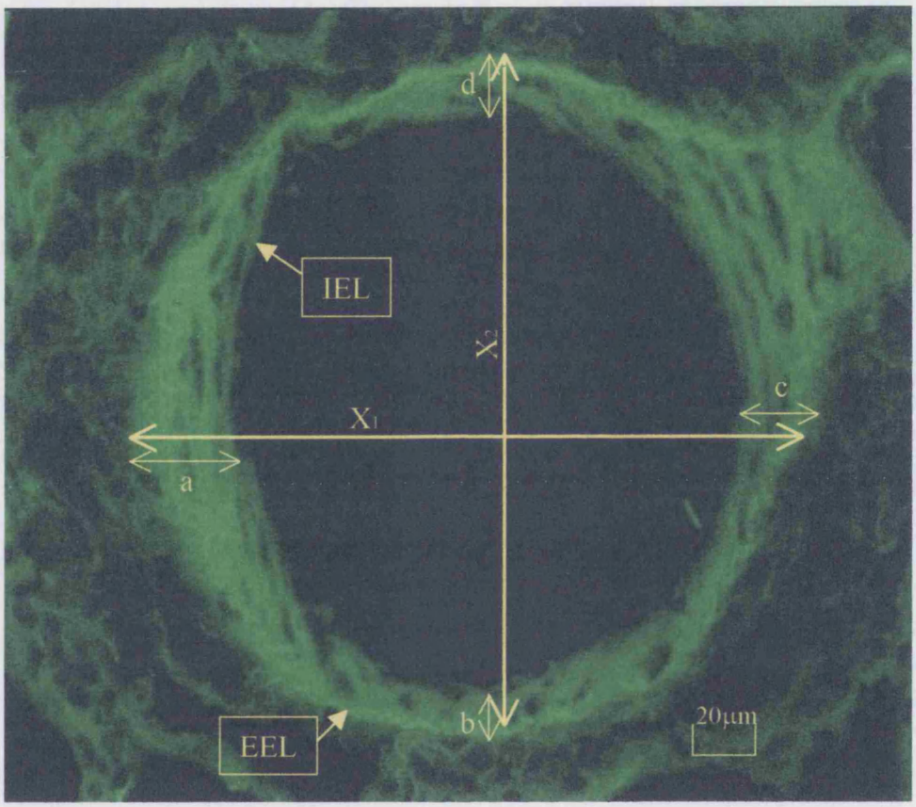

Figure 2.4: Measurement of pulmonary artery under microscope

Pulmonary vascular diameter (X_1 and X_2) and wall thickness (a,b,c,d) were identified by external elastic laminae (EEL) and internal elastic laminae (IEL), ×400 magnification.

distance between two diametrically opposed external elastic laminae (Figure 2.4); two measurements were made: one along the line corresponding to the longest distance and another one along the line perpendicular to it, and these two values were averaged to get mean arterial diameter. Medial wall thickness was defined and measured as the distance between the internal and the external elastic laminae; four measurements were made: one in each of the four quadrants defined by the perpendicular lines. The percentage wall thickness is calculated from the formula (Hislop & Reid, 1976):

Wall thickness (%) = 2 × Medial wall thickness × 100/ Diameter

All pulmonary arteries distinguished under the microscope were measured only if they were entire ones; around 60 vessels were measured from each rat.

2.7 Isolated pulmonary artery ring

2.7.1 Procedure of setting up

Male Wistar rats (200-250g) were killed by cervical dislocation. The heart and lung *en bloc* was removed and placed in Krebs' solution. Pulmonary arteries were always obtained from the upper left lobe to avoid variation. The left pulmonary arteries (ID: 1059 ± 95μm, n = 7) and the fourth order branches (ID: 234 ± 20μm, n = 7) of pulmonary arteries were dissected in Krebs' solution under a dissection microscope. The bronchiole was the guide for tracing the pulmonary arteries because the bronchiole always overlaps

on the top of the pulmonary artery from the ventral side of the lobe. Isolated pulmonary arteries were cleared of connective tissue and cut into 2mm lengths. The vessels were mounted in a dual Mulvany-Halpern myograph using two tungsten wires (25μm diameter, Goodfellow Co. UK). One tungsten wire was attached to a micrometer, the other one attached to an isometric transducer (Grass Instruments Company, USA). The vessels were incubated in Krebs' solution bubbled with 20%O_2/5%CO_2/ 75%N_2 at 37°C, and stretched from zero to 1mN and equilibrated for 1 hour.

2.7.2 Normalization of pulmonary artery on myograph

Following an equilibration, the passive length-tension relationship of the vessel was determined, called normalization (Mulvany & Halpern, 1977; Teng & Barer, 1995).

A) Internal circumference (IC):

$$\mathbf{IC\ (\mu m) = (X_1 - X_0) \times 2 + (2 + \Pi) \times D}$$

X_0 (μm) is the micrometer reading when the two wires on the myograph just touch each other and bring the tension to zero. X_1 (μm) is the micrometer reading when the vessel was successively stretched and a tension was given to the vessel. D is the diameter of the tungsten wire used for mounting the vessels.

B) Internal diameter (ID):

$$\mathbf{ID\ (\mu m) = IC / \Pi}$$

C) The Laplace's equation to determine the transmural pressure (P):

$$\mathbf{P\ (kPa) = T / \{2L \times IC / (2\ \Pi \times 1000)\}}$$

T (mN) is the tension reading on the myograph when stretching the vessel at the micrometer reading at X_1; L is the length of vessel ring (2mm). P is converted to mmHg if divided by 0.1333.

The resting tension was raised to give an equivalent transmural pressure of 12 - 16 mmHg to the pulmonary artery rings in normoxic rats, approximately reflecting *in vivo* transmural pressures in pulmonary artery (McCulloch & MacLean, 1995). The resting tension was raised to an equivalent pressure of 25 - 30 mmHg for the pulmonary artery rings in CH rats (McCulloch *et al.*, 1996).

Isometric tension responses to agonists were recorded through a Maclab computer system. At each concentration the tension was allowed to increase to a plateau before changing to the next concentration. 50mM KCl was added first to test the viability of smooth muscle. Vessels were precontracted with 1μM PHE and then followed by 3μM ACH to test the viability of the endothelial cells. Agonist-induced vasoconstrictor responses were standardized by expressing them as a percentage of the vasoconstrictor response to 50mM KCl in the same vessel. Cumulative concentration-dependent contractions to KCl, PHE, Ang II were recorded in the fourth order branches of

pulmonary arteries and/or the left pulmonary arteries. Vessels were thoroughly washed between each set of contractile responses to agonists.

2.8 Permeabilized pulmonary and mesenteric artery preparations

2.8.1 α-Toxin

α-Toxin which was isolated from Staphylococcus aureus strain Wood 46, can bind to cell membranes to form hydrophilic pores (1 – 2 nm diameter). These hydrophilic pores permit the passage of small molecules (up to 1kDa) but prevent the loss of essential cytoplasmic proteins from the cells. Therefore, integral regulatory components such as calmodulin- and cyclic nucleotide-dependent protein kinases are maintained at physiological concentrations (Ohanian *et al.*, 1997).

Permeabilised arteries allow $[Ca^{2+}]_i$ to be controlled by EGTA, a Ca^{2+} chelator, and yet receptors remain coupled to their intracellular signalling pathways. It provides a useful method for studying Ca^{2+} mobilization and Ca^{2+} sensitization. With the $[Ca^{2+}]_i$ clamped and the intracellular stores depleted, any observed vasoconstriction could be only due to an increased sensitivity of the contractile apparatus to Ca^{2+} (Evans *et al.*, 1999).

2.8.2 Permeabilization procedure

Male Wistar rats (200 - 250g) were killed by cervical dislocation. The heart and lung *en*

bloc and a segment of the ileum with attached mesentery were removed and placed in Krebs' solution. Pulmonary arteries were always obtained from upper left lobe to avoid variation. The fourth order branches of pulmonary arteries (2 mm length) and the third order branches of mesenteric arteries (2 mm length) were dissected in Krebs' solution under a microscope. One long tungsten wire was passed through the vessel lumen for handling the vessels. The wire was bent at right angles at both sides to prevent the tissue slipping off.

The vessels were permeabilized with α-toxin (1000 haemolytic units/ml) in 10mM EGTA solution (called relaxing solution), and incubated in Eppendorf tubes for 30 min. The vessel with tungsten wire was removed from α-toxin solution and mounted on a myograph in relaxing solution and gassed with 95% O_2/5% CO_2 at room temperature (22 - 25°C) for 30min without any initial tension. The tension on the permeabilized vessels was set up at 1mN before drug administration (Evans *et al.*, 1999; Hill *et al.*, 2000).

2.8.3 Demonstration of permeabilization

Permeabilization was assessed by vasoconstriction responses to Ca^{2+} (10nM - 10μM), these concentrations of Ca^{2+} had no effect on the intact vessels. Ca^{2+} concentration-tension curves were obtained by mixing relaxing solution and Ca^{2+} EGTA solution (called contracting solution) or adding $CaCl_2$ to 80nMCa^{2+}/ 0.2mM EGTA solution (called refilling solution). Figure 2.5 shows a representative tracing of Ca^{2+}-tension responses in a permeabilized pulmonary artery ring.

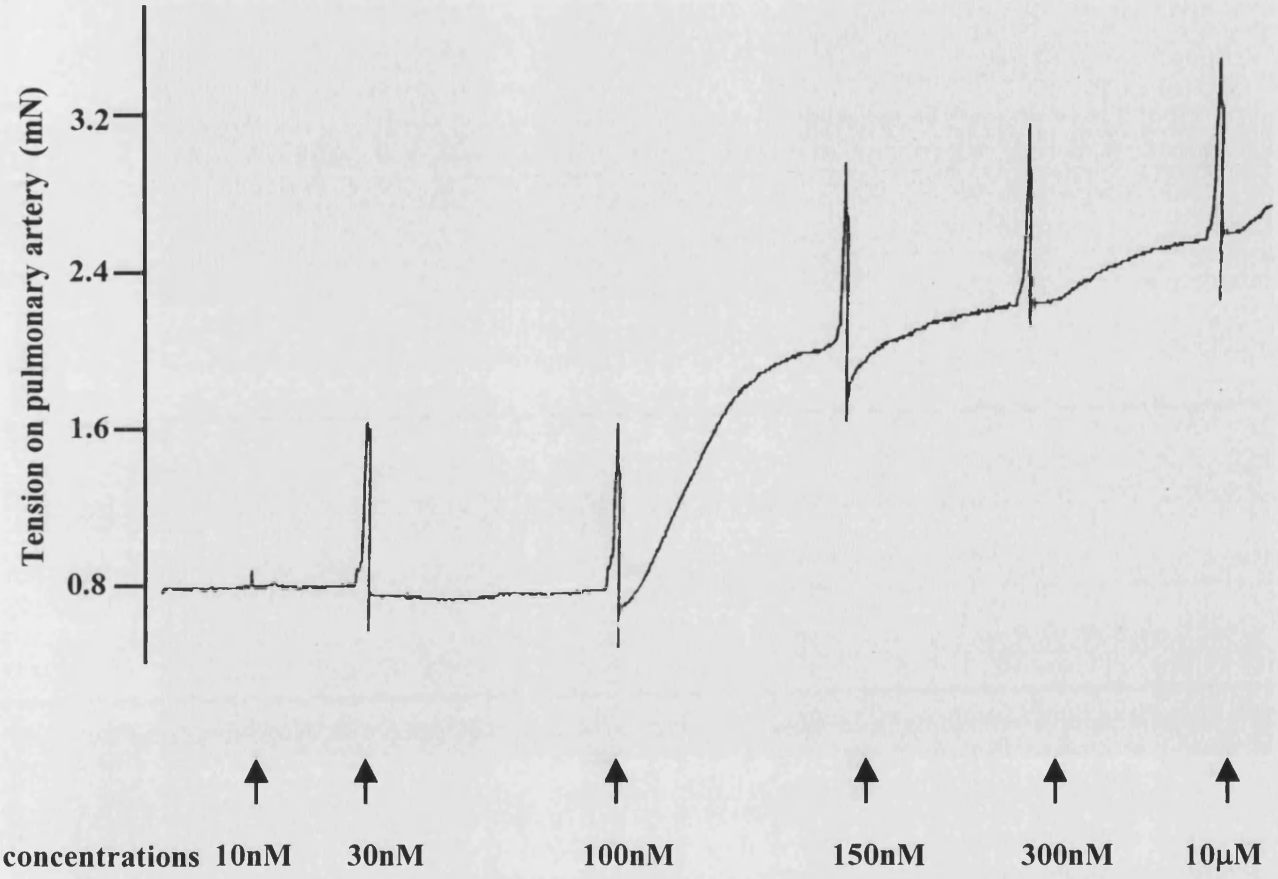

Figure 2.5 Demonstration of permeabilization in the pulmonary artery

Ca^{2+} (10nM-10 μM) induced the vasoconstriction in the permeabilized pulmonary artery. Up to 10 μM, Ca^{2+} did not induce any vasoconstriction in non-permeabilized pulmonary artery. Ca^{2+} was buffered by relaxing and contracting solutions. The peak responses are artefacts from changing solutions.

A computer programme adapted from Fabiato and Fabiato (1979) by Dr. G.D. Smith (University of Glasgow) was used to calculate the equilibration concentrations of free metal ions and affinity constants relating to ionic strength and pH. Affinity constants for H^+, Ca^{2+} and Mg^{2+} binding to EGTA were taken from Smith and Miller (1984). $[Ca^{2+}]_i$ in mock intracellular solutions were estimated from the proportion of relaxing solution and contracting solution, according to equation:

$$\mathbf{[Ca^{2+}]_i = K_d \times [relaxing\ solution/contracting\ solution]}$$

K_d (affinity constant) for EGTA associated with Ca^{2+}, which is influenced by temperature, pH, ionic strength and concentrations of heavy metals, was calculated using a computer program.

Examining the ability of intracellular Ca2+ stores in arteries to refill with the mock intracellular solution of minimal Ca2+ content (80nM Ca2+/0.2mM EGTA) also assessed the effectiveness of permeabilization. 10mM caffeine was applied to the arterial segment maintained in the mock intracellular solution and following wash out the caffeine was re-applied at 10min intervals. A repeatable caffeine-evoked contractile response, corresponding to intracellular store Ca^{2+} release, was taken to demonstrate complete permeabilisation (Hill *et al.*, 2000).

2.9 Drugs and chemicals

Drugs were obtained from the following sources: ET-1 was obtained from Peptide Institute (Japan). BQ123 (cyclo[D-Asp-L-Pro-D-Val-L-Leu-D-Trp]) and BQ788 ([N-cis-2,6-dimethylpiperidinenocarbonyl-L-rMe-Leu-D-Trp (COOMe)-D-Nle-Ona]) were supplied by Rhone-Poulenc Rorer (Dagenham, England). PD156707 (sodium 2-benzo(1,3)dioxol-5-yl-4-(4-methoxy-phenyl)-4-oxo-3-(3,4,5-trimethoxy-benzyl)-but-2-enoate) was a generous gift from Parke-Davis Pharmaceutical Research, Ann Arbor, Michigan, USA. HOE642 (4-isopropyl-3-methyl-sulphonylbenzoyl-guanidine methanesulphonate) was a generous gift from Dr. H. J. Lang, Hoechst Pharmacuiticals. 5-HT, PHE, NA, Adr, heparin sulphate, Ang II, HEPES (N-2-hydroxyethylpiperaxine-N-2-ethanesulphonic acid), creatine phosphate, EGTA (ethylene glycol-bis(b-aminoethylether)-N,N,N',N'-tetra acetic acid), prazosin, U46619 (9, 11-dideoxy-11a, 9a-epoxymethano-prostaglandin F2α) and ATP (adenosine 5'-triphosphate) were obtained from Sigma company. K^+ proprionate was obtained from Phase Separation Ltd. Staphylococcus aureus α-toxin (haemolysin) and Ro-32-0432 (2-{8-[(dimethylamino)methyl]-6,7,8,9-tetrahydropyrido[1,2-a]indol-3-yl}-3-(1-methylindol-3-yl)maleimide, hydrochloride were obtained from Calbiochem. The chemicals for Krebs' solution were obtained from BDH.

NA and Adr were dissolved in distilled water with ascorbic acid (10^{-4} M). ET-1 was dissolved in normal saline and stored at –20°C. BQ123, PD17567, staurosporine and corticosterone were dissolved in DMSO; the final concentration of DMSO in perfusate

was less than 0.1%. U46619 was dissolved in 95% ethanol and stored at –80°C. α-Toxin was dissolved in distilled water and stored at 4°C. Caffeine (100 mM) was prepared in refilling solution. All other drugs were dissolved in distilled water.

2.10 Appendices

2.10.1 Krebs' solution

	Concentration (mM)
Potassium chloride	4.7
Potassium dihydrogen phosphate	1.2
Calcium chloride	1.25
Magnesium sulphate	1.2
Sodium chloride	118
Sodium bicarbonate	25
Glucose	11.1

Krebs' solution was prepared freshly. Potassium chloride, potassium dihydrogen phosphate, calcium chloride and magnesium sulphate were taken from stock solutions.

2.10.2 Mock intracellular solutions

INGREDIENTS	Relaxing solution	Contracting solution	Refilling solution	Final concentration
EGTA/Ca^{2+}EGTA	100mM×10ml EGTA	100mM×10ml Ca^{2+}EGTA	100mM×0.2ml EGTA	10mM for relaxing and contracting solutions, 0.2mM for refilling solution
$CaCl_2$			0.01M×94.6µl	80nM
HEPES	500mM×5ml	500mM×5ml	500mM×5ml	25mM
$MgCl_2$	700mM×1ml	700mM×1ml	700mM×1ml	7mM
K propionate	1M×10ml	1M×10ml	1M×10ml	100mM
Na_2ATP	0.312g	0.312g	0.312g	5M
Creatine PO4	0.504g	0.504g	0.504g	15mM
Distilled Water	73.5ml	73.5ml	83.3ml	
Total Volume	100ml	100ml	100ml	

The pH of the mock intracellular solutions was adjusted to 7.0 with 1M KOH.

2.10.3 Stock solutions for mock intracellular solutions

COMPOUNDS	M.W.	CONCENTRATION	VOLUME (ml)
EGTA	368.98	100mM	100ml
Ca^{2+}EGTA		100mM (EGTA)	100ml
HEPES	238.3	500mM	100ml
$MgCl_2$	203.3	700mM	100ml
K^+ propionate	130	1M	100ml
KOH	56.11	1M	100ml

When preparing EGTA (100mM×100ml) stock solution, 3.6898g EGTA was dissolved in 20ml 1M KOH first, adding a small amount at a time meanwhile swirling it. 1M KOH was added if needed to fully dissolve EGTA.

When preparing Ca^{2+}EGTA (100mM × 100ml) stock solution, 1.08g $CaCO_3$ was added in 100mM × 100ml EGTA stock solution. $CaCO_3$ was added gradually whilst swirling contents of beaker above low burning bunsen burner until bubbles (CO_2) had come off and the solution was clear.

HEPES, $MgCl_2$(6H2O), potassium propionate and potassium hydroxide were prepared with distilled water. Na_2ATP (MW605.19) and creatine PO_4 (MW 255.1) were added freshly in relaxing solution or contracting solution.

2.11 Statistics

All responses were plotted against doses/concentrations of vasoconstrictors on a logarithmic scale, using Origin (2.94 version) computer program. The potencies of drugs were expressed as ED_{50} which is the dose producing 50% of the maximum response to a particular agent.

All data shown were means ± s. e. means. Data were analyzed by unpaired Student's t-test or paired Student's t-test for two independant samples or comparison between paired data respectively. One-way analysis of variation (ANOVA) with Dunnett's test was applied to data from more than two groups. The significance was accepted if $P < 0.05$. The statistics program used is MiniTab.

CHAPTER THREE

RESULTS

3.1 Pulmonary vascular reactivity during normoxia and CH

3.1.1 Effect of CH on indices of pulmonary hypertension

As indices of hypoxic pulmonary hypertension, haematocrit, basal PPP and ratio of right ventricular weight/total ventricular weight were investigated in CH rats and the weight-matched control rats.

As shown in Figure 3.1, haematocrit significantly increased from 43.2 ± 2.0% in the controls to 57.2 ± 1.0% in CH rats, $P < 0.001$, $n = 10 - 13$. Three weeks of hypoxia resulted in significant increases in the basal PPP from 6.3 ± 0.9mmHg in the controls to 10.5 ± 0.2mmHg in CH rats, $P < 0.01$, $n = 5$ (Figure 3.2).

Figure 3.3 shows the ratio of right ventricular weight to total ventricular weight in the controls and CH rats. The ratio significantly increased from 0.21 ± 0.01 in the control rats to 0.33 ± 0.01 in CH rats, $P < 0.001$, $n = 13 - 15$.

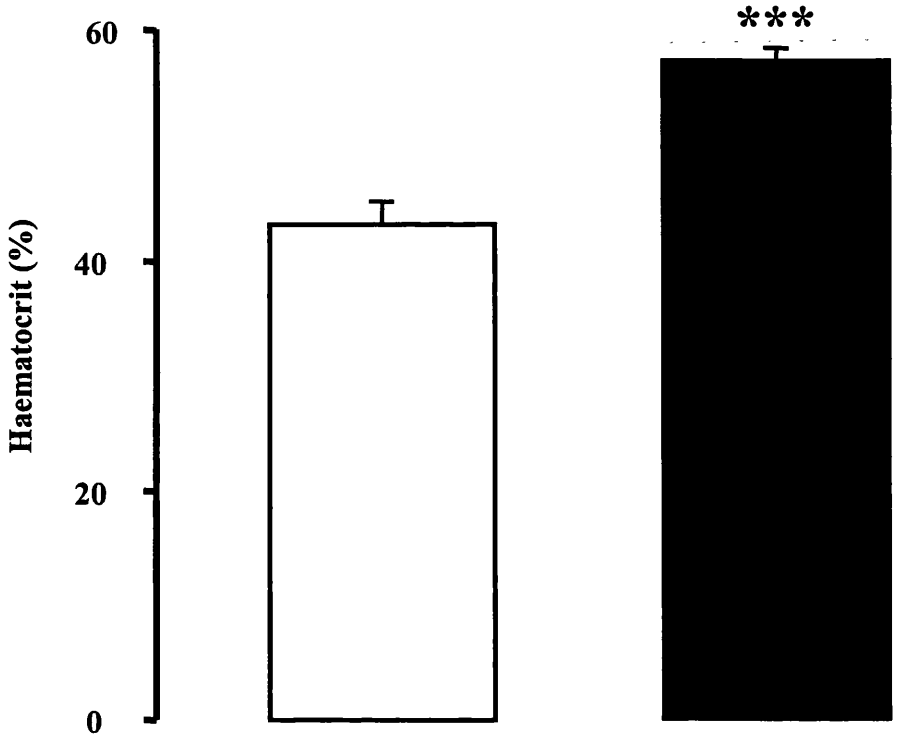

Figure 3.1: Haematocrit in the normoxic and CH rats.

Blood samples were collected from the normoxic rats as the controls (□), weight-matched to CH rats (■). Each column represents mean ± s. e. mean. ***P < 0.001 vs. the controls; unpaired Student's t-test. n = 10 for the control group and n = 13 for the CH group.

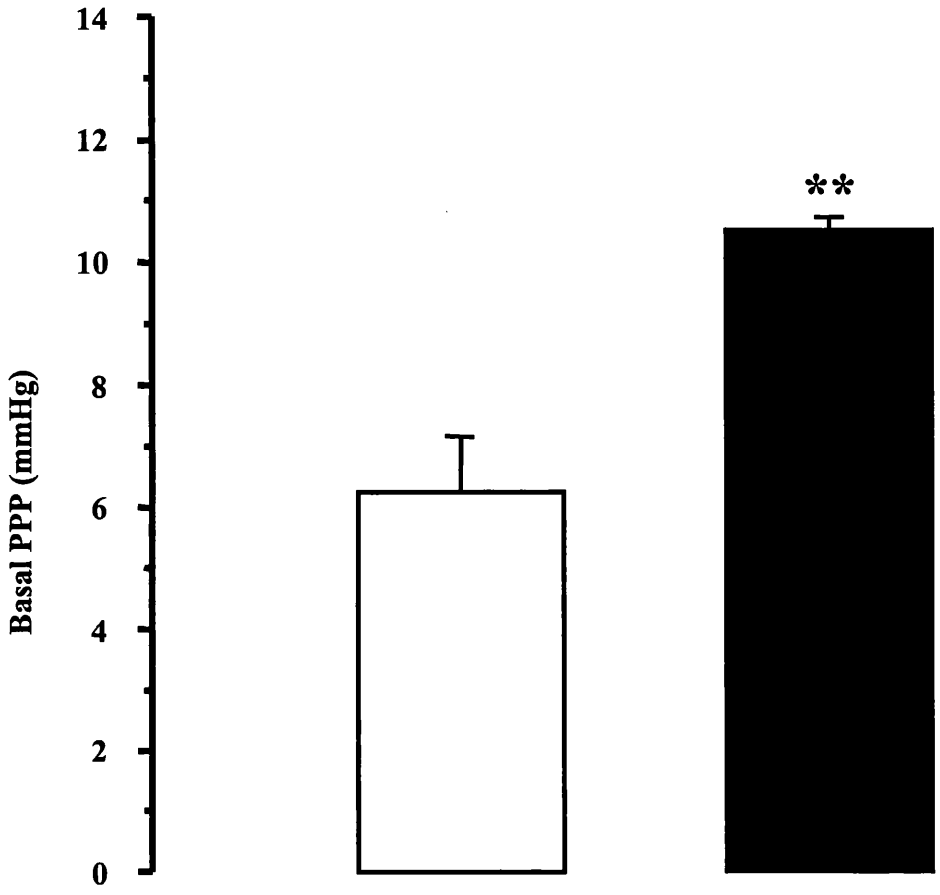

Figure 3.2: Basal PPP during normoxia and CH in the isolated perfused lungs.

Basal PPP in CH rats (■) was significantly higher than in the control group (□). Each column represents mean ± s. e. mean. *P < 0.01 vs. the normoxic group; unpaired Student's t-test. n = 5 for each group.

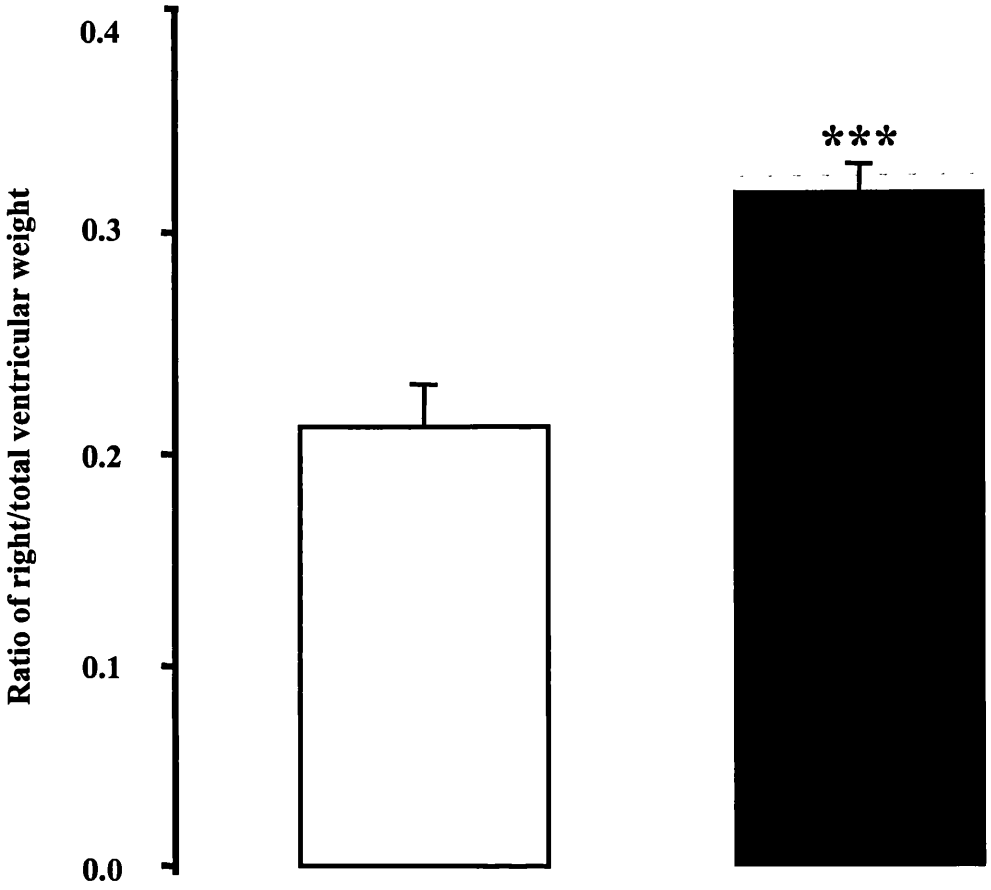

Figure 3.3: Index of right ventricular hypertrophy in the normoxic and CH rats.

The ratio of right ventricular weight to total ventricular weight was taken as index of right ventricular hypertrophy in the normoxic (□) and CH (■) rats. Each column represents mean ± s. e. mean. *** $p < 0.001$vs. the controls; unpaired Student's t-test. n = 13 for the control group and n = 15 for the CH group.

3.1.2 Effect of CH on responses to PHE in isolated lungs

PHE was bolus-injected into the isolated perfused lungs through the pulmonary artery, and PPP was recorded in both CH lungs and the weight-matched controls. The vasoconstrictor responses to all doses of PHE (1 - 30 nmoles) were significantly enhanced in CH isolated perfused lungs when compared to the controls (Figure 3.4). For example, 10 nmoles PHE induced increases of PPP in the controls of 2.7 ± 0.3 mmHg (n = 7) and 15.4 ± 3.0 mmHg (n = 5) in CH rats, $P < 0.01$. However ED_{50} values of the two groups were not significantly different.

In the isolated aortic rings, cumulative concentration-dependent contraction curves of PHE were built up from the weight-matched controls and CH rats. The contractile responses to PHE were not enhanced in the CH group (n = 7), compared to the control group (n = 4) (Figure 3.5).

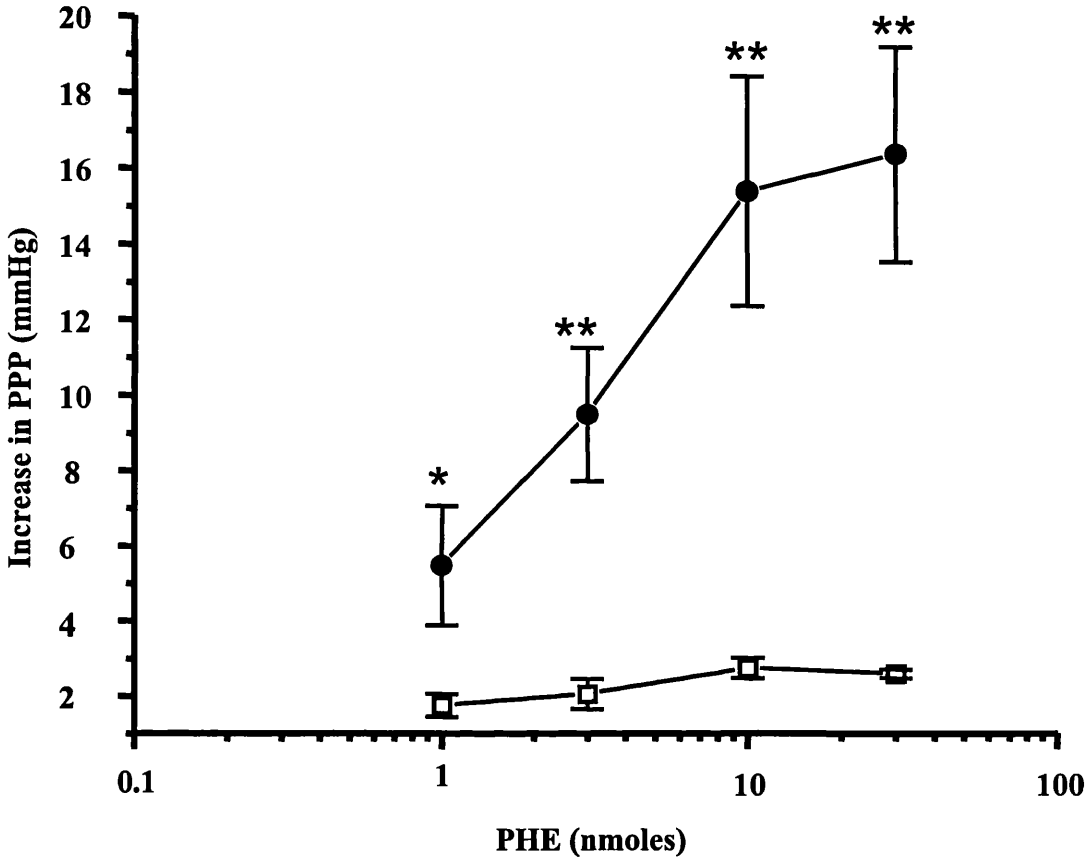

Figure 3.4: Vasoconstrictor responses to PHE in normoxic and CH isolated perfused lungs.

Data are presented as increases of PPP (mmHg) to bolus injections of PHE (1 - 30 nmoles) into the lungs from normoxic (□) and CH (●) animals. Each point represents mean ± s. e. mean. *P< 0.05 vs. the controls; **P < 0.01 vs. the controls; unpaired Student's t-test. n = 9 for the normoxic rats and n = 11 for the CH rats.

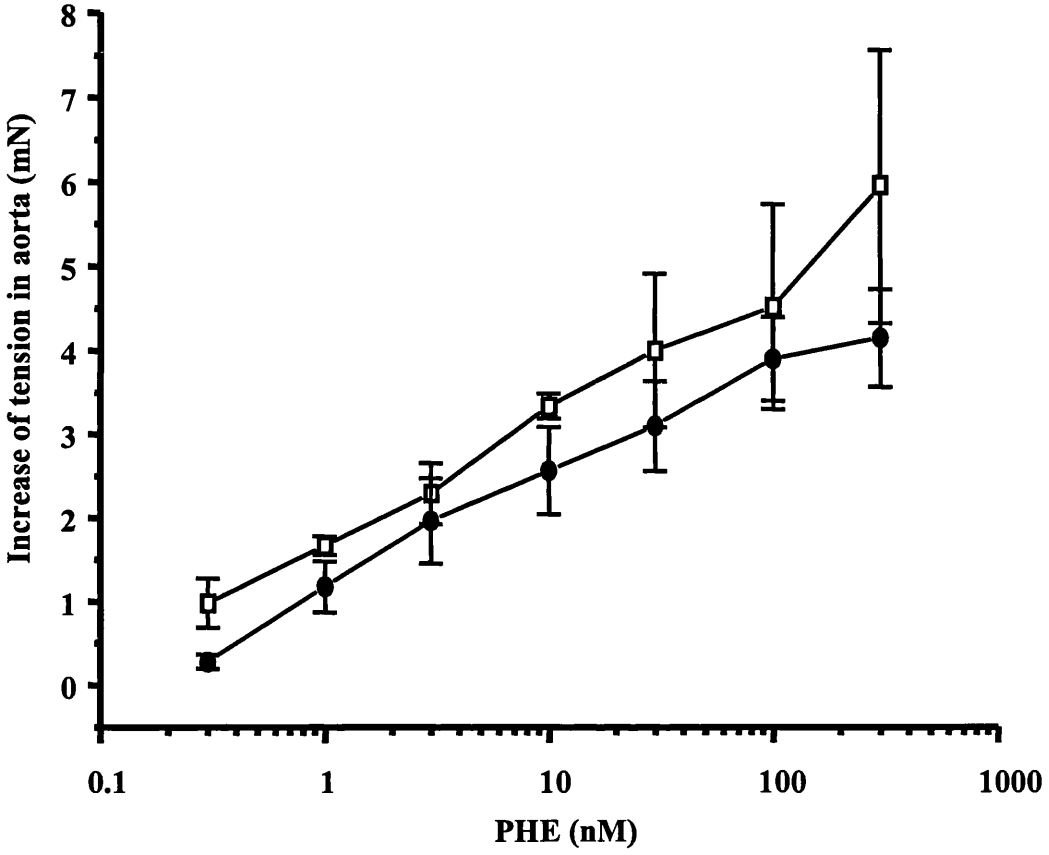

Figure 3.5: Concentration-dependent contractions to PHE in normoxic and CH isolated aortic rings.

Data are presented as increases of tension (mN) to cumulative concentrations of PHE (0.3 - 300 nM) in the aortic rings from normoxic (□) and CH (●) rats. Each point represents mean ± s. e. mean. n = 4 for the normoxic rats and n = 7 for the CH rats.

3.1.3 Effect of different oxygen tensions on responses to PHE in CH isolated lungs

The isolated lungs from CH rats were perfused with Krebs' solution bubbled with low oxygen (10% O_2/85% N_2/5% CO_2). Low oxygen tension did not significantly change the vasoconstrictor responses to PHE in CH isolated perfused lungs, compared to the results from the group gassed with 20% O_2/75% N_2/5% CO_2 ($P > 0.05$) (Figure 3.6).

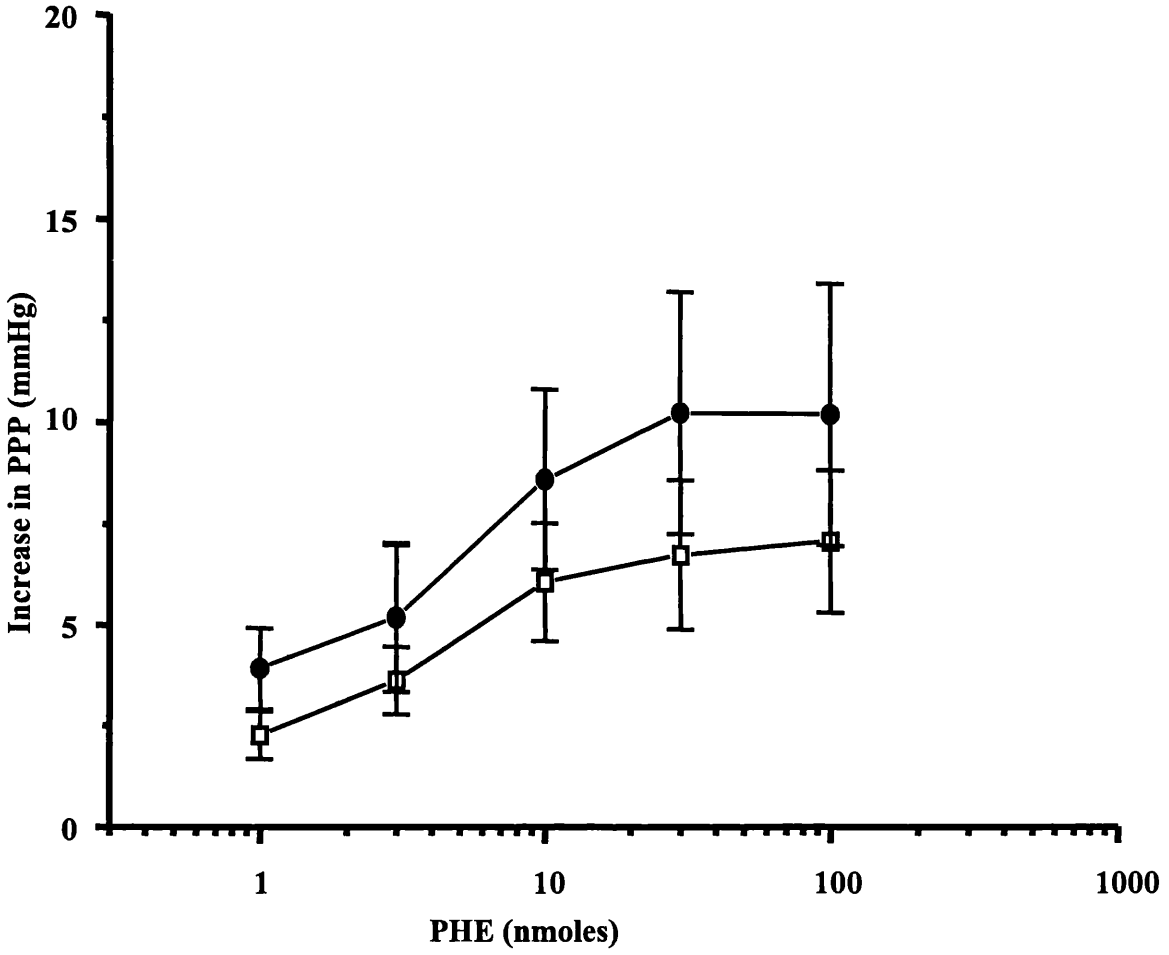

Figure 3.6: Effect of different oxygen tensions on vasoconstrictor responses to PHE in CH isolated perfused lungs.

Data are presented as increases of PPP (mmHg) to bolus injections of PHE (1 - 100 nmoles) into the CH lungs perfused with 20% O_2 (□) or 10% O_2 (●). Each point represents mean ± s. e. mean. n = 6 for each group.

3.1.4 Effects of ET-1 receptor antagonists and ET converting enzyme inhibitor on responses to PHE in CH isolated lungs

It was decided to see whether vascular synthesis of ET-1 modulated the vasoconstrictor responses to PHE during CH. ET_A receptor antagonists, an ET_B receptor antagonist and an ET converting enzyme inhibitor were used.

To confirm that the concentration of PD156707 (5 μM) was sufficient to block ET_A receptors on the pulmonary vascular smooth muscle, the vasoconstrictor responses to ET-1 were recorded in the absence and presence of PD156707. PD156707 (5 μM) obviously inhibited the ET-1-induced vasoconstrictor responses in normoxic isolated perfused lungs (Figure 3.7). However, PD15670 did not affect the vasoconstrictor responses to PHE in CH isolated lungs (Figure 3.8).

BQ788 had no effect on the basal PPP. Also BQ788 did not affect PHE-induced vasoconstrictor responses in CH isolated perfused lungs (Figure 3.9). PPP increases induced by 30nmoles PHE in the absence and presence of BQ788 (5 μM) were 8.4 ± 2.3 mmHg and 6.9 ± 1.4, respectively ($n = 5$, $P > 0.05$).

Phosphoramidon (10 μM), an ET converting enzyme inhibitor also had no effect on the vasoconstrictor responses to PHE in CH isolated perfused lungs ($P > 0.05$, $n = 4$) (Figure 3.10).

The time course of PPP increases to PHE in CH isolated perfused lungs was also studied (Figure 3.11). Dose-responses to PHE were repeated after a 15 min interval, which is the same period as used for infusion of the antagonists. The two dose-response curves of PHE were quite similar, $P > 0.05$, $n = 4$.

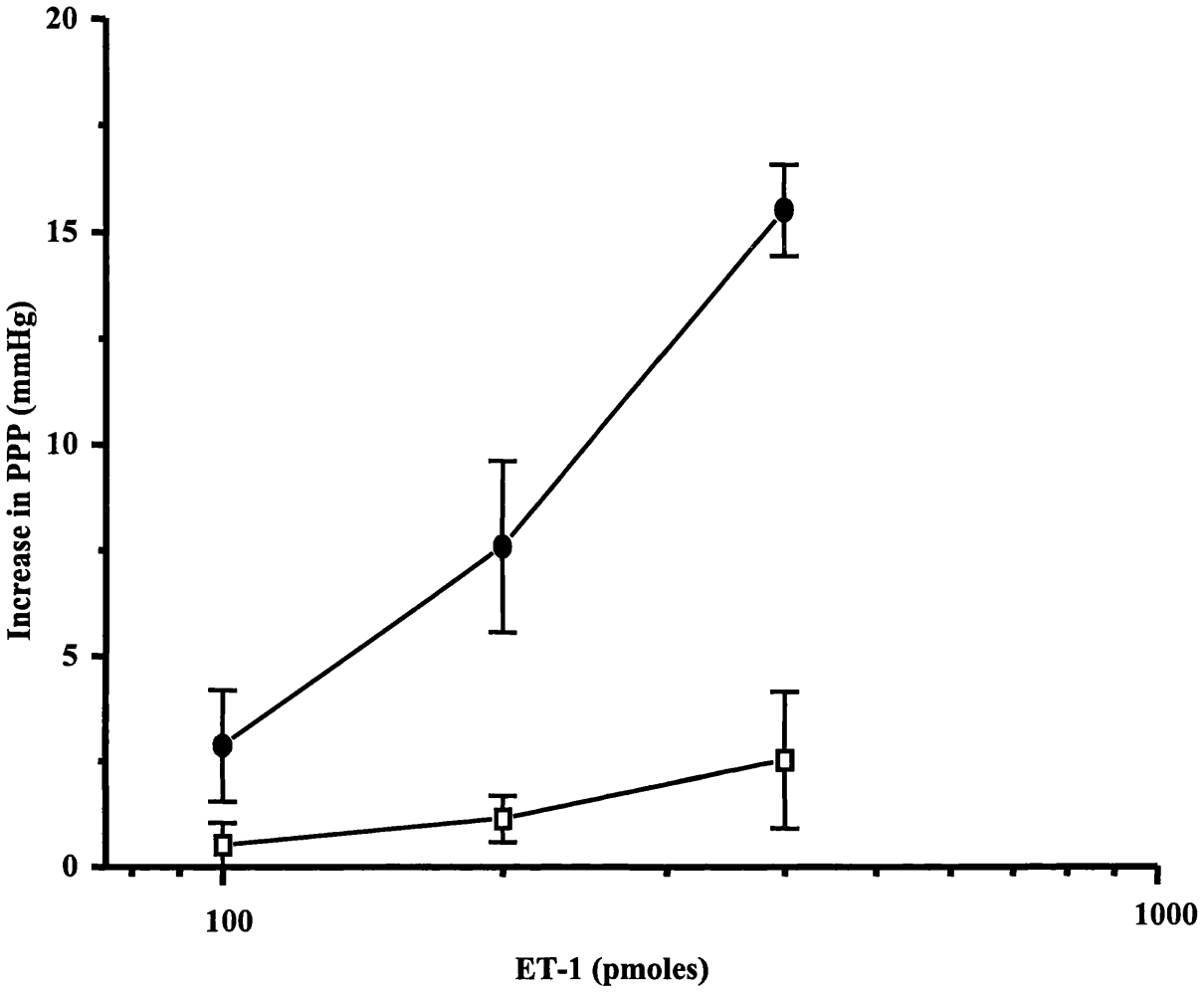

Figure 3.7: Effect of a selective ET_A receptor antagonist, PD156707 on the vasoconstrictor responses to ET-1 in the normoxic isolated perfused lungs.

The vasoconstrictor responses to ET-1 (100 - 400 pmoles) were recorded in the absence (●) or presence (□) of PD156707 (5 μM) in the normoxic isolated perfused lungs. Each point represents mean ± s. e. mean, n = 2 for each group.

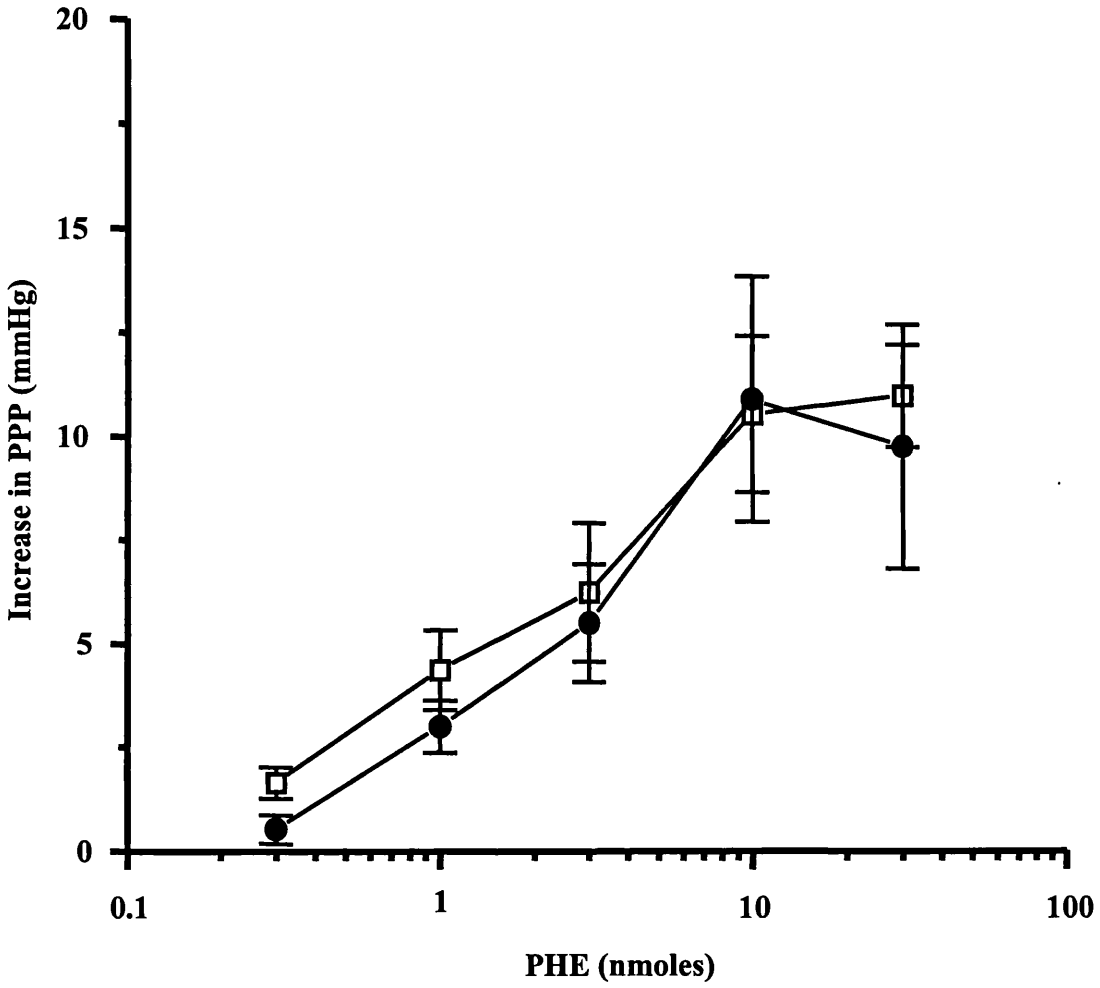

Figure 3.8: Effect of a selective ET_A receptor antagonist, PD156707 on the vasoconstrictor responses to PHE in CH isolated perfused lungs.

The vasoconstrictor responses to PHE (0.3 - 30 nmoles) were recorded in the absence (□) or presence (●) of PD156707 (5 μM) in CH lungs. Each point represents mean ± s. e. mean; n = 4 for each group.

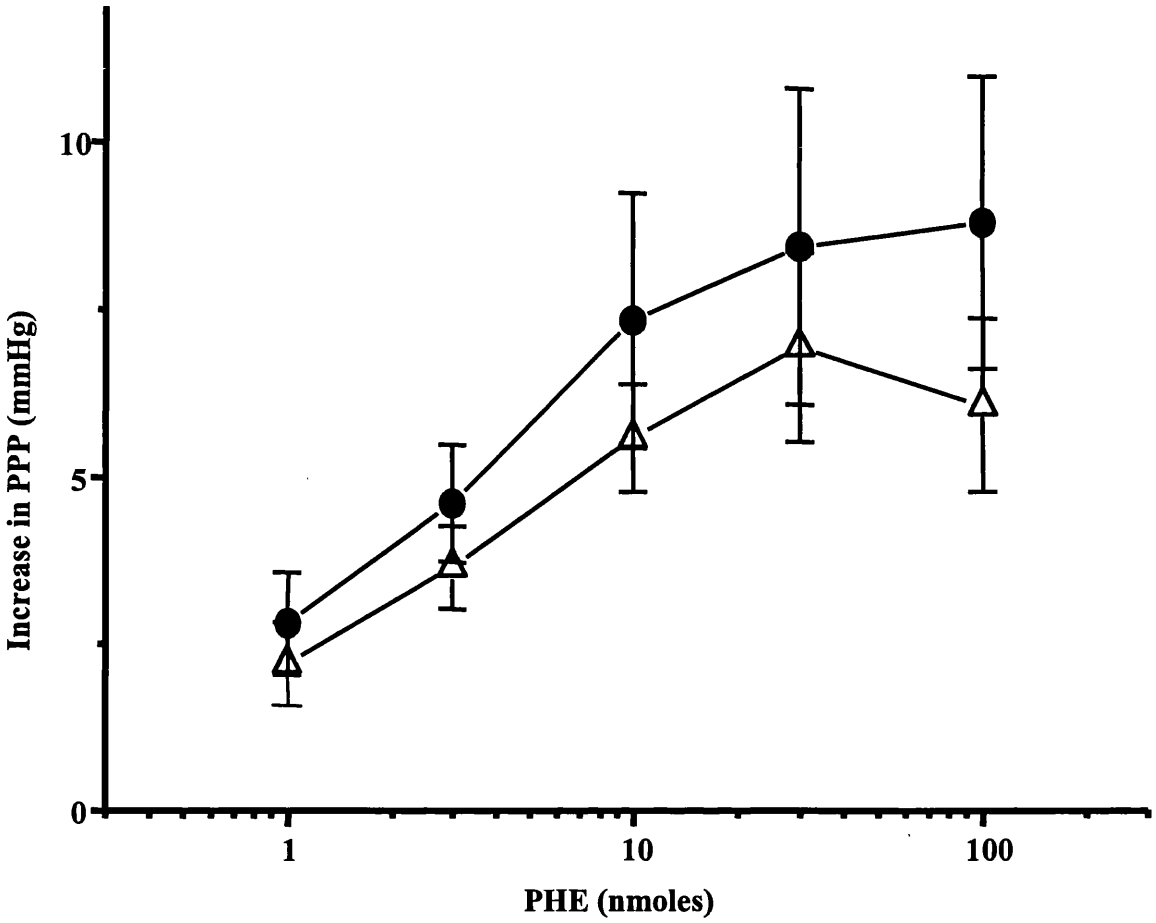

Figure 3.9: Effect of a selective ET_B receptor antagonist, BQ788 on the vasoconstictor responses to PHE in CH isolated perfused lungs.

The vasoconstrictor responses to PHE (1 - 100 nmoles) were recorded in the absence (●) or presence (△) of BQ788 (5 μM) in CH lungs. Each point represents mean ± s.e.mean, n = 5 for each group.

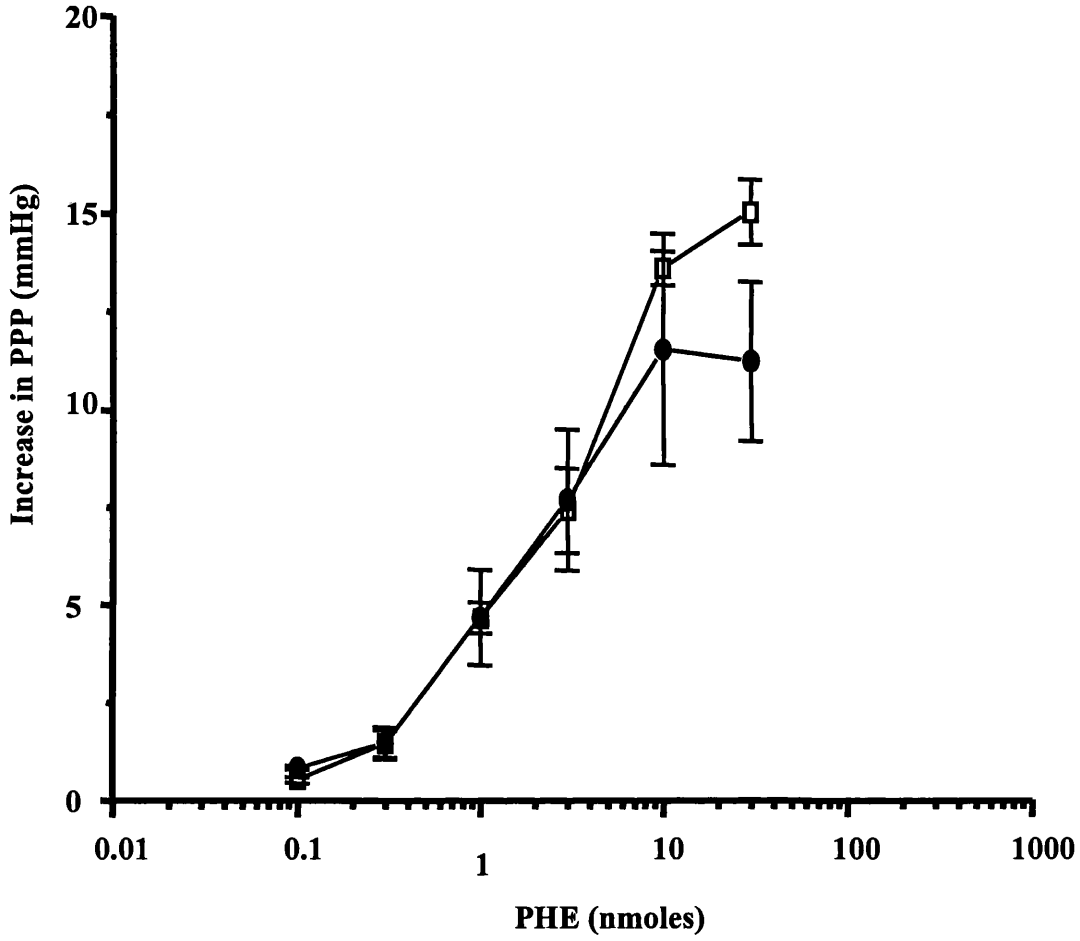

Figure 3.10: Effect of an ET converting enzyme inhibitor, phosphoramidon on the vasoconstrictor responses to PHE in CH isolated perfused lungs.

The vasoconstrictor responses to PHE (0.1 - 30 nmoles) were recorded in the absence (□) or presence (●) of phosphoramidon (10 μM) in CH isolated perfused lungs. Each point represents mean ± s. e. mean. n = 4 for each group.

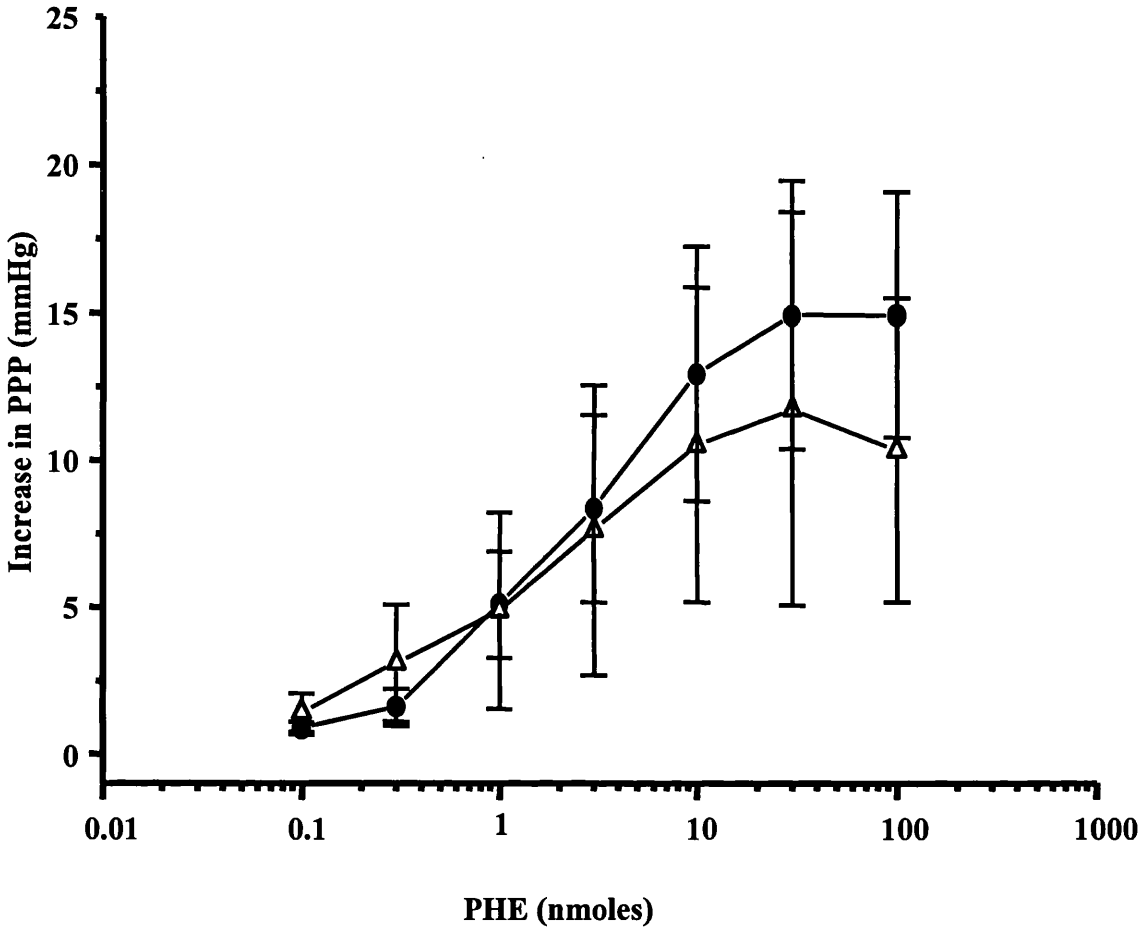

Figure 3.11: Reproducibility of the vasoconstrictor responses to PHE in CH isolated perfused lungs.

Bolus injections of PHE (0.1 - 100 nmoles) into CH lungs were repeated after a 15min interval (△) and compared with the original controls (●). Each point represents mean ± s.e.mean. n = 4 for each group.

3.1.5 Effect of CH on responses to other agonists in isolated lungs

In order to see whether the enhanced vasoconstrictor responses in the CH isolated perfused lungs could be universal, a series of experiments were performed to investigate the effect of CH on the magnitude of vasoconstrictor responses to other agonists in isolated perfused lungs.

Table 3.1 summarizes increases in PPP induced by agonists in normoxic and CH isolated perfused lungs. All substances tested induced potentiated responses in CH isolated perfused lungs when compared with the controls, especially at high doses. But there is no difference in ED_{50} values of the controls and CH lungs for any agonist.

Table 3.1: Effect of CH on the vasoconstrictor responses in isolated perfused lungs.

Agonists	Doses	PPP increase (mmHg)		ED_{50}	
		Control (number)	CH (number)	Control (number)	CH (number)
PHE	10 nmol	2.4 ± 0.7 (9)	5.7 ± 0.8 (10)**	4.4 ± 1.4 nmol (8)	5.4 ± 0.9 nmol (10)
NA	1 nmol	2.7 ± 0.5 (6)	8.8 ± 1.9 (5)*	0.3 ± 0.01 nmol (5)	0.4 ± 0.1 nmol (5)
Ang II	300 pmol	8.0 ± 1.4 (4)	23.6 ± 4 (4)*	45 ± 2.9 pmol (3)	46 ± 5 pmol (4)
U46619	30 pmol	3.2 ± 1.1 (5)	7.1 ± 0.6 (3)*		
KCl	200 μmol	8.5 ± 1.2 (6)	18.7 ± 3.8 (5)*		

Data are presented as increases of PPP (mmHg) to bolus injections of agonists into the lungs from normoxic and CH animals. PPP increases induced by all agonists were potentiated in CH isolated perfused lungs, compared to the controls. Due to pulmonary oedema at high doses of KCl and U46619 the maximal responses and ED50 values to these two agonists were not obtained.
Each group of data represents mean ± s. e. mean. *$P < 0.05$ vs. the controls; **$P < 0.01$ vs. the controls; unpaired Student's t-test.

3.1.5.1 Effect of CH on responses to Ang II in isolated lungs

Ang II (1 – 300 pmoles) was tested in CH and control lungs. CH exposure potentiated Ang II-induced vasoconstrictor responses, compared to the controls (Figure 3.12). The threshold responses between CH and control lungs were not obviously different, only at high doses the vasoconstrictor responses to Ang II in CH lungs were enhanced. For example, 300 pmoles Ang II induced increases in PPP as 8.0 ± 1.4 mmHg in the controls and 23.6 ± 4.6 mmHg in CH lungs, $P < 0.05$, $n = 3 - 4$.

BQ123 (5 μM), which reduced the potentiated vasoconstrictor responses to PHE in CH perfused lungs, had no effect on the vasoconstrictor responses to Ang II in the CH lungs ($P > 0.05$, $n = 4$) (Figure 3.13).

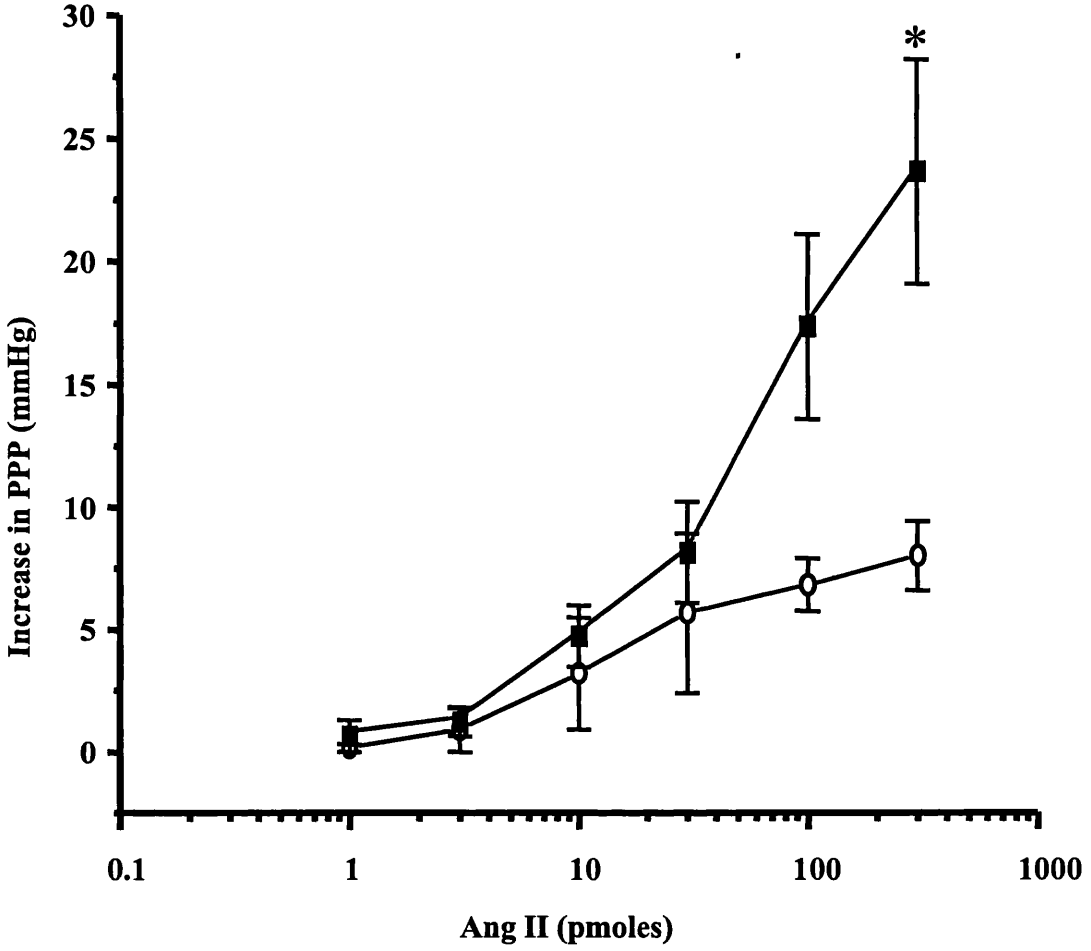

Figure 3.12: Vasoconstrictor responses to Ang II in normoxic and CH perfused lungs.

Data are presented as increases of PPP (mmHg) to bolus injections of Ang II (1 - 300 pmoles) into the lungs from normoxic (O) or CH (■) animals. Each point represents mean ± s. e. mean. *P< 0.05 vs. the controls; unpaired Student's t-test. n = 4 for each group.

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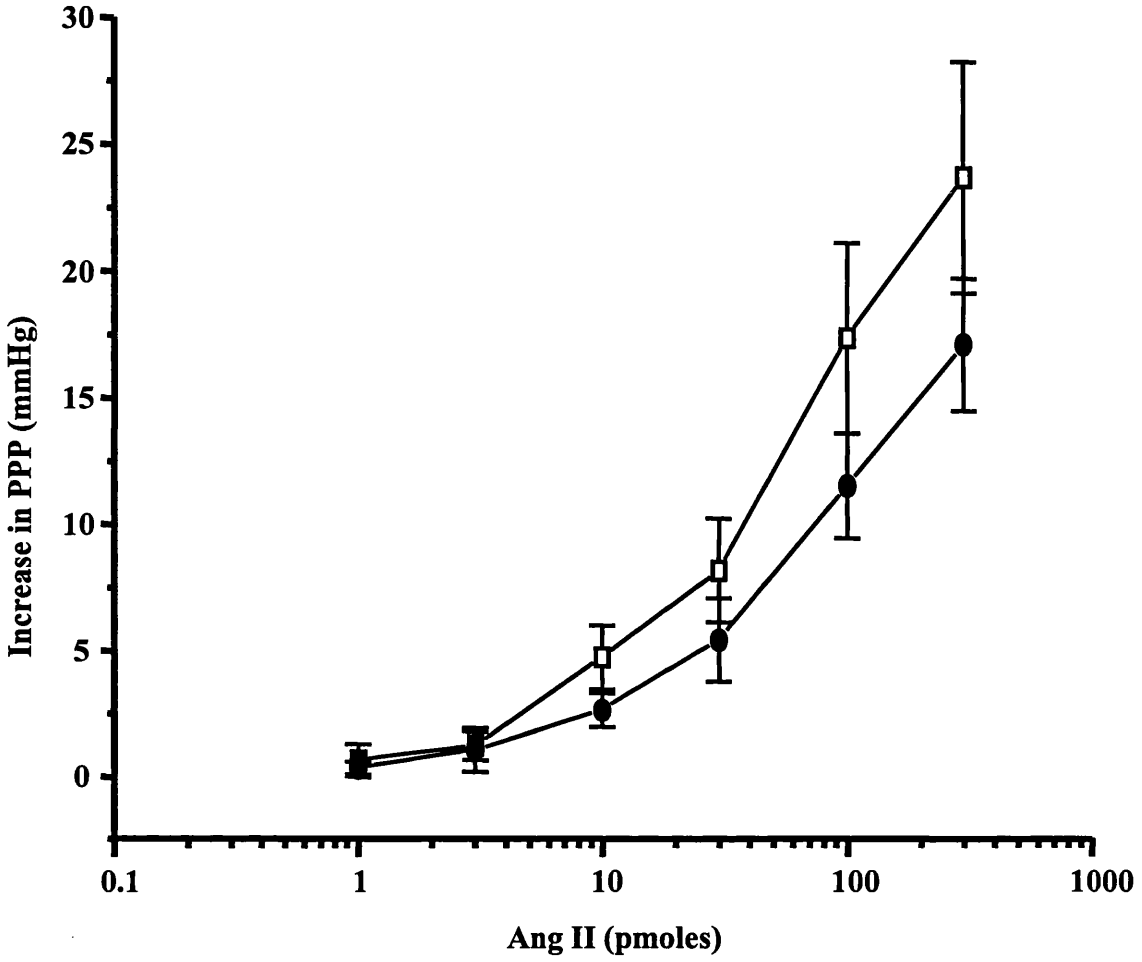

Figure 3.13: Effect of an ET_A receptor antagonist, BQ123 on the responses to Ang II in CH isolated perfused lungs.

The vasoconstrictor responses to Ang II (1 - 300 pmoles) were recorded in the absence (□) or presence (●) of BQ123 (5 μM) in CH lungs. Each point represents mean ± s. e. mean, n = 4 for each group.

3.1.5.2 Effect of CH on responses to KCl in isolated lungs

KCl (25 – 400 μmoles) was also tested in isolated perfused lungs. CH significantly increased KCl-induced vasoconstrictor responses in isolated perfused lungs (Figure 3.14). 200 μmoles KCl increased PPP from 8.5 ± 1.2 mmHg in the controls to 18.7 ± 3.8 mmHg in the CH lungs, $P < 0.05$, $n = 5 - 6$. The threshold doses of KCl in CH and the control group were not different. The response to maximal dose of KCl was not recorded because the isolated perfused lungs became oedematous quickly with high doses of KCl.

The vasoconstrictor responses to KCl in CH lungs were not affected by BQ123 (5 μM) or BQ788 (5 μM) (Figure 3.15).

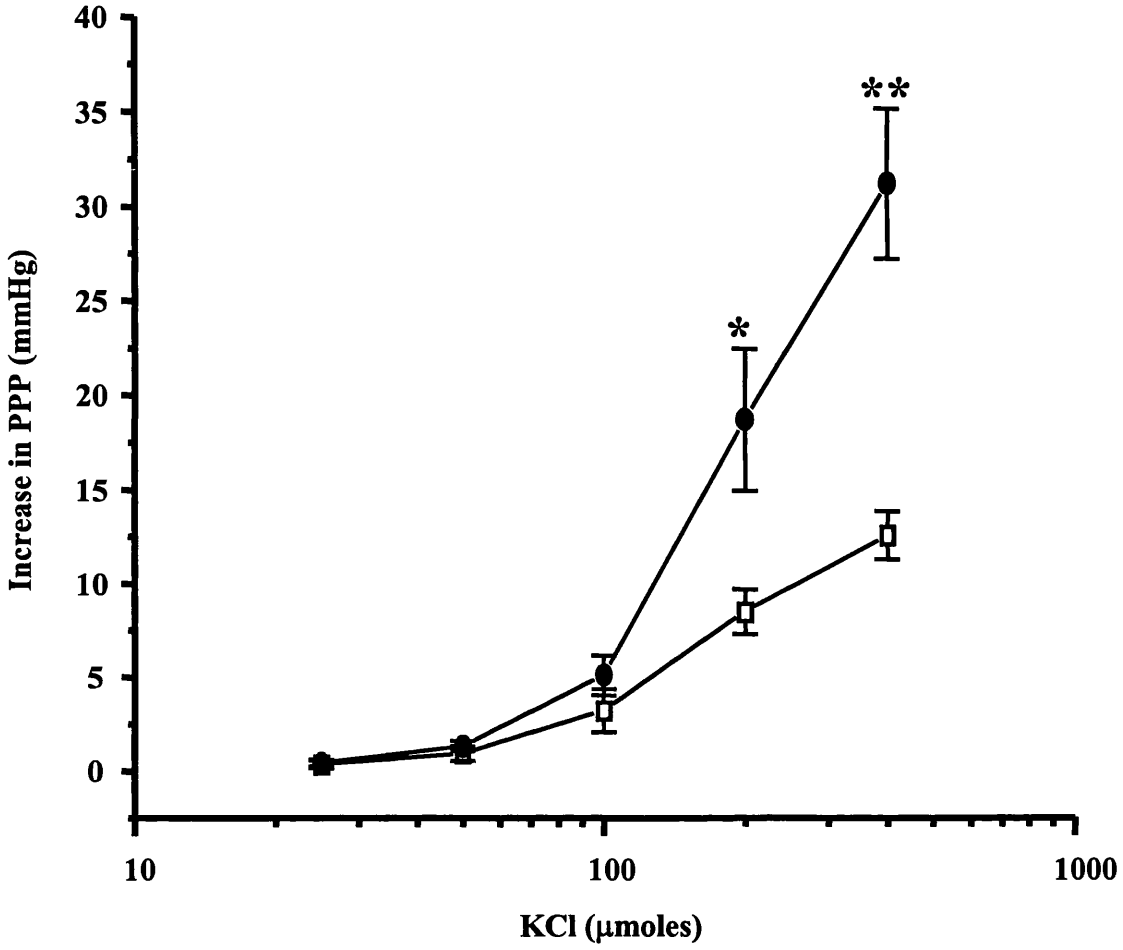

Figure 3.14: Vasoconstrictor responses to KCl in normoxic and CH perfused lungs.

Data are presented as increases of PPP (mmHg) to bolus injections of KCl (25 - 400 μmoles) into the lungs from the normoxic (□) and CH (●) animals. Each point represents mean ± s. e. mean. *P < 0.05 vs. the controls; **P < 0.01 vs. the controls; unpaired Student's t-test. n = 5 - 6 for the normoxic and CH rats.

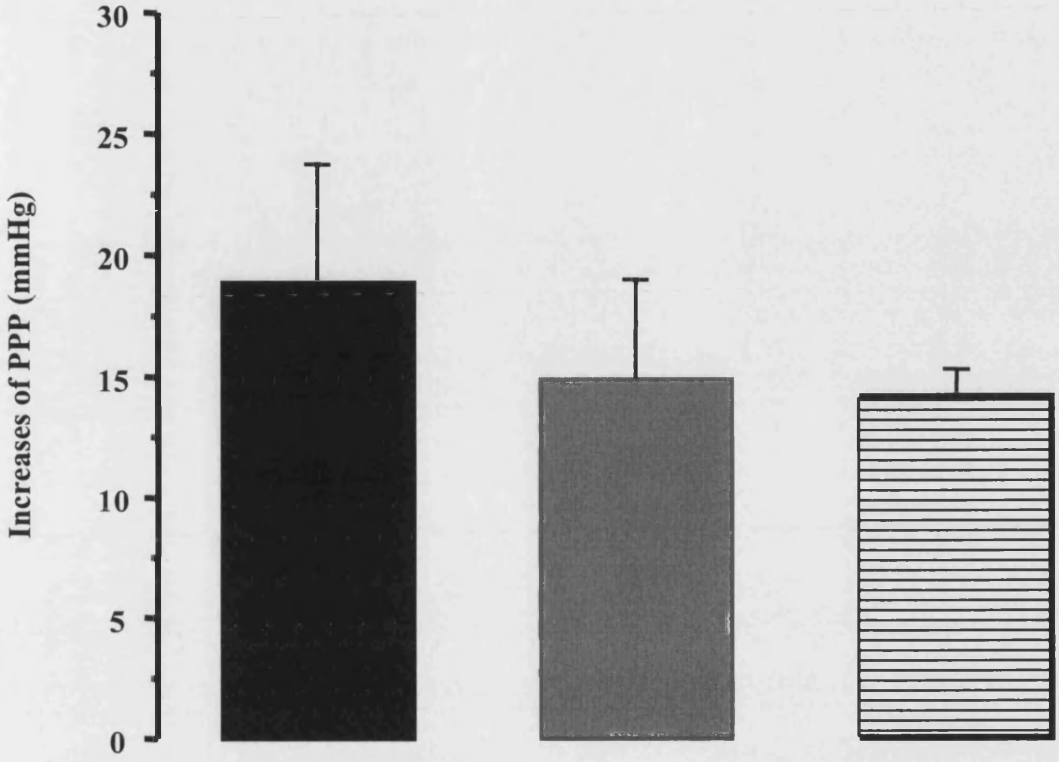

Figure 3.15: Effect of ET_A receptor antagonist, BQ123 and ET_B receptor antagonist, BQ788 on KCl-induced vasoconstrictor responses in CH isolated perfused lungs.

The vasoconstrictor responses to KCl (200 μmoles) were recorded in the controls (■) or in the presence of BQ123 (5 μM) (■) or BQ788 (5 μM) (▤) in CH isolated perfused lungs. Each column represents mean ± s. e. mean. n = 4 for each group.

3.1.5.3 Effect of CH on responses to NA and U46619 in isolated lungs

Figure 3.16 shows the vasoconstrictor responses to NA in the isolated perfused lungs. Similar to PHE, NA-induced vasoconstrictor responses were potentiated in the isolated perfused lungs from CH animals, compared to the controls. 1 nmole NA produced increases in PPP of 2.7 ± 0.5 mmHg in the controls and 8.8 ± 1.9 mmHg in the CH lungs, respectively ($P < 0.05$, $n = 5 - 6$).

The vasoconstrictor responses to U46619 (1 – 30 pmoles) were also potentiated in CH perfused lungs (Figure 3.17). 30 pmoles U46619 increased PPP of 3.2 ± 1.1 mmHg in the controls vs. 7.1 ± 0.6 mmHg in the CH lungs ($P < 0.05$, $n = 3 - 5$). The response to maximal dose of U46619 was not recorded because the isolated perfused lungs became oedematous quickly with high doses of U46619.

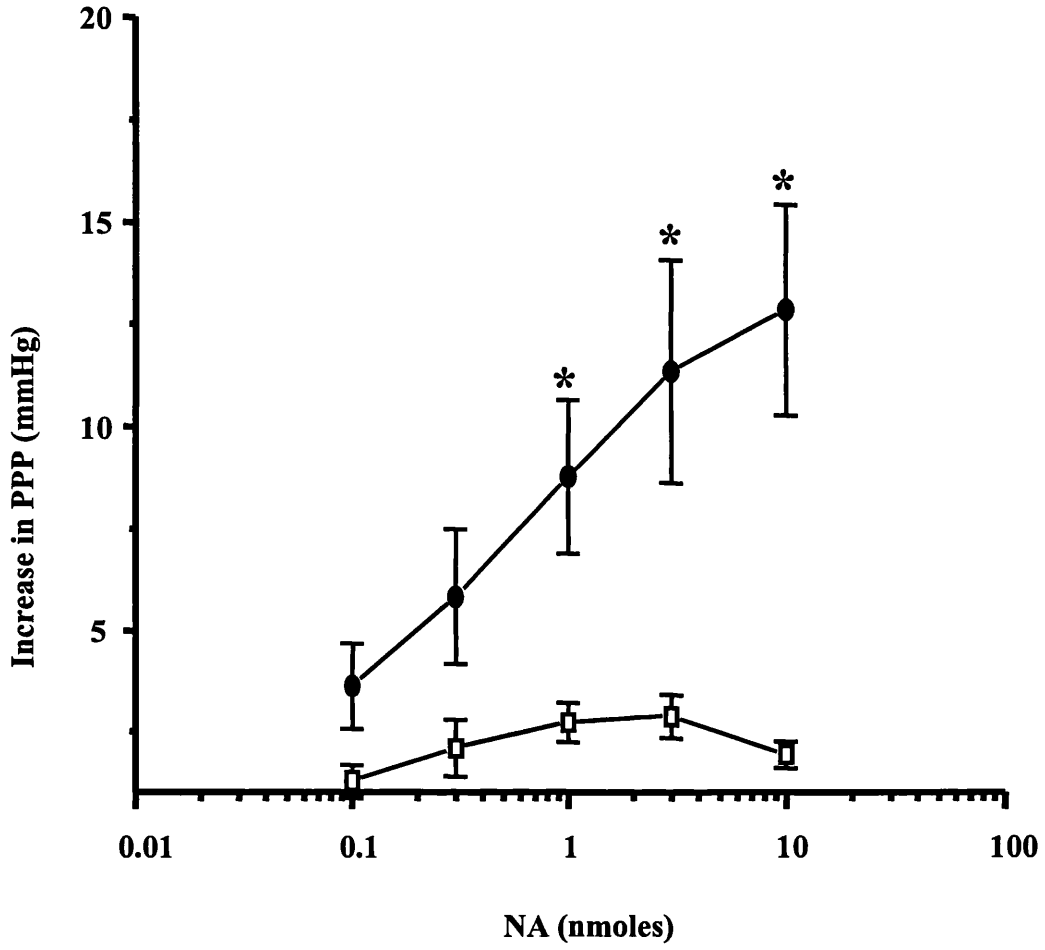

Figure 3.16: Vasoconstrictor responses to NA in the normoxic and CH perfused lungs.

Data are presented as increases of PPP (mmHg) to bolus injections of NA (0.1 - 10 nmoles) into the lungs in normoxic (□) and CH (●) animals. Each point represents mean ± s. e. mean. *P < 0.05 vs. the controls; unpaired Student's t-test. n = 6 for the normoxic rats and n = 5 for the CH rats.

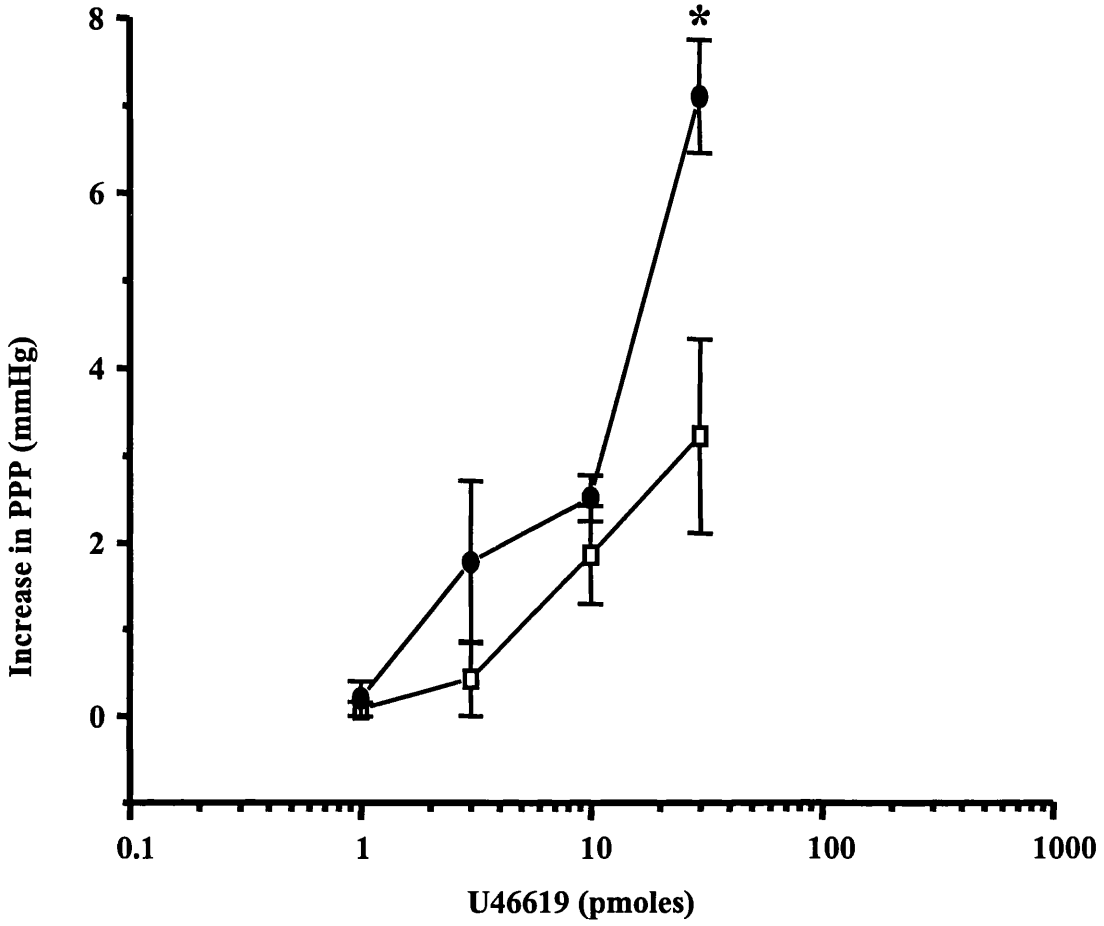

Figure 3.17: Vasoconstrictor responses to U46619 in normoxic and CH perfused lungs.

Data are presented increases of PPP (mmHg) to bolus injections of U46619 (1 - 30 pmoles) into lungs in normoxic (□) and CH (●) animals. Each point represents mean ± s. e. mean. *P < 0.05 vs. the controls; unpaired Student's t-test. n = 5 for the normoxic rats and n = 3 for the CH rats.

3.1.6 Comparison of vascular reactivity between large and small pulmonary arteries

As a large pulmonary artery, the left pulmonary artery (ID: 1059 ± 95 μm, n = 7) was chosen and the fourth order branch of the pulmonary artery (ID: 234 ± 20 μm, n = 7) was taken as a small pulmonary artery. The vessels were mounted on wire myograph and the basal tension was set up at 4.1 ± 0.3 mN (n = 7) for the large pulmonary arteries and 1.1 ± 0.1 mN (n = 9) for the small pulmonary arteries, mimicking the transluminal tension in the pulmonary artery of 12 - 16mmHg *in vivo*.

KCl induced concentration-dependent contractions in both large and small pulmonary arteries. Contraction to 50 mM KCl, a sub-maximal concentration was chosen for standardising the vasoconstrictor responses to all vasoconstrictors in the same preparation. The absolute values of vasoconstrictor responses to KCl are greater in the large pulmonary arteries than the small pulmonary arteries. However, there was no difference in standardised concentration-dependent contraction curves to KCl in the large and small pulmonary arteries (Figure 3.18).

PHE induced concentration-dependent contractions in the large pulmonary arteries. However, in the small pulmonary arteries, much smaller responses were observed. The maximal contraction to PHE (1 μM) was 83 ± 10 % (n = 6) in the large pulmonary arteries and 24.2 ± 9.3 % (n = 6) in the small pulmonary arteries, $P < 0.01$ (Figure 3.19).

Ang II contracted in both sizes of the pulmonary artery rings. In contrast with the responses to PHE, the vessels desensitised to Ang II very quickly (Figure 3.20). The contractile responses to Ang II were diminished in the small pulmonary arteries compared to the large ones, although there was no significant difference between the response curves in the large and small pulmonary arteries.

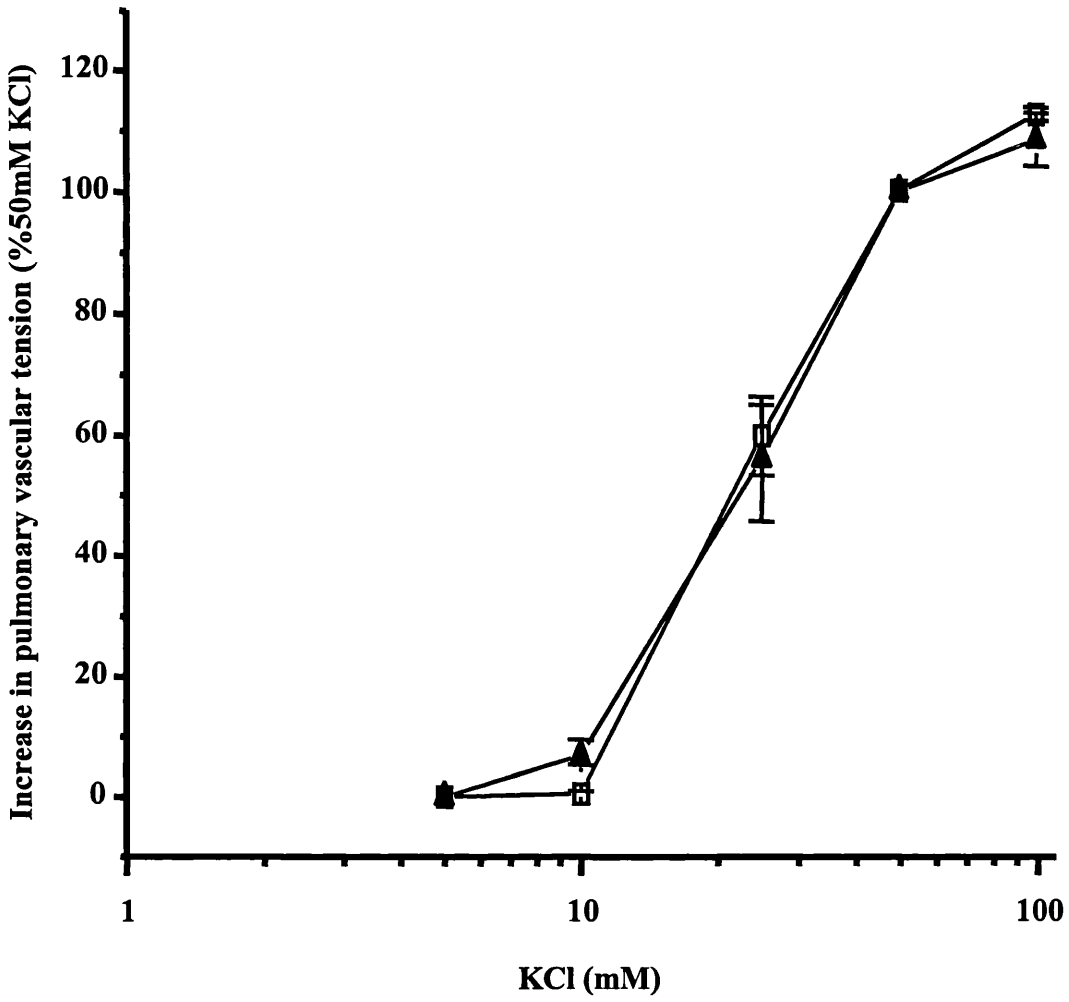

Figure 3.18: Concentration-dependent contractions to KCl in large and small pulmonary arteries from normoxic rats.

Vascular responses to KCl (5 - 100 mM) in the large (□) or small (▲) pulmonary artery rings are expressed as a percentage of contraction to 50 mM KCl in the same preparation. Each point represents mean ± s. e. mean. n = 6 for the large pulmonary arteries and n = 8 for the small pulmonary arteries.

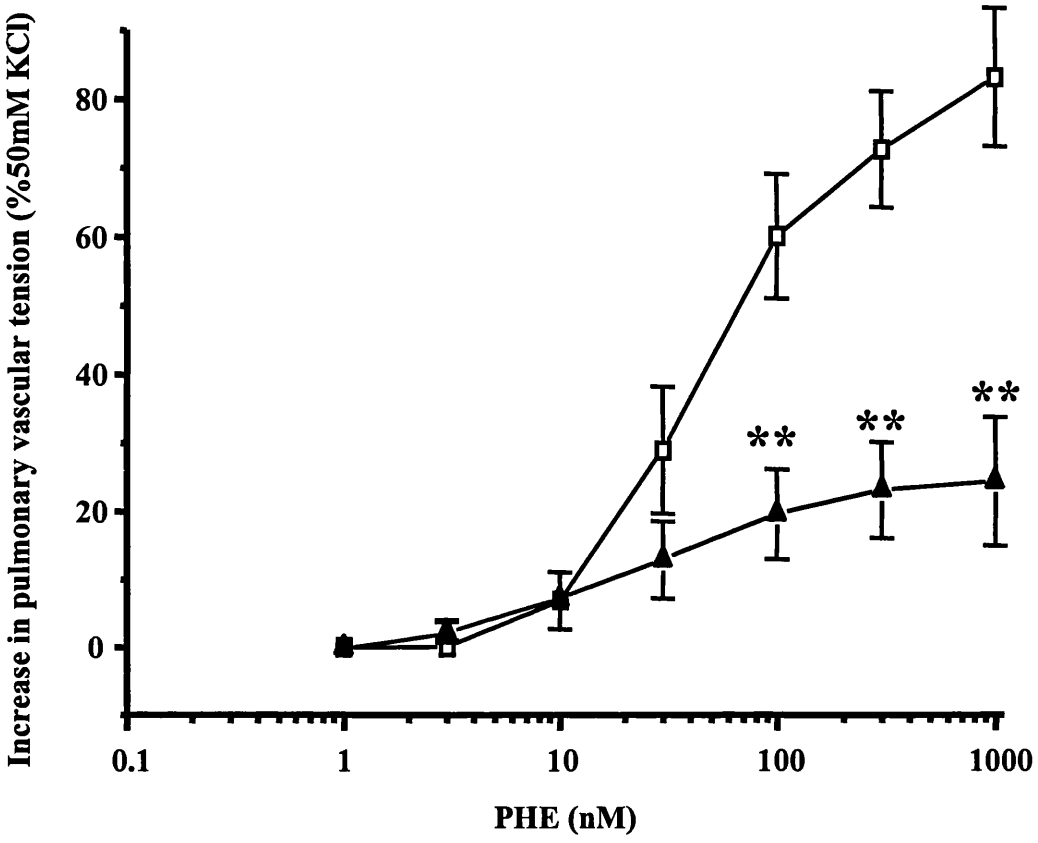

Figure 3.19: Concentration-dependent contractions to PHE in large and small pulmonary arteries from normoxic rats.

Vascular responses to PHE (1 - 1000 nM) in large (□) or small (▲) pulmonary artery rings are expressed as a percentage of contraction to 50 mM KCl in the same preparation. Each point represents mean ± s. e. mean. **P < 0.01 vs. the large pulmonary arteries; unpaired Student's t-test. n = 6 for each group.

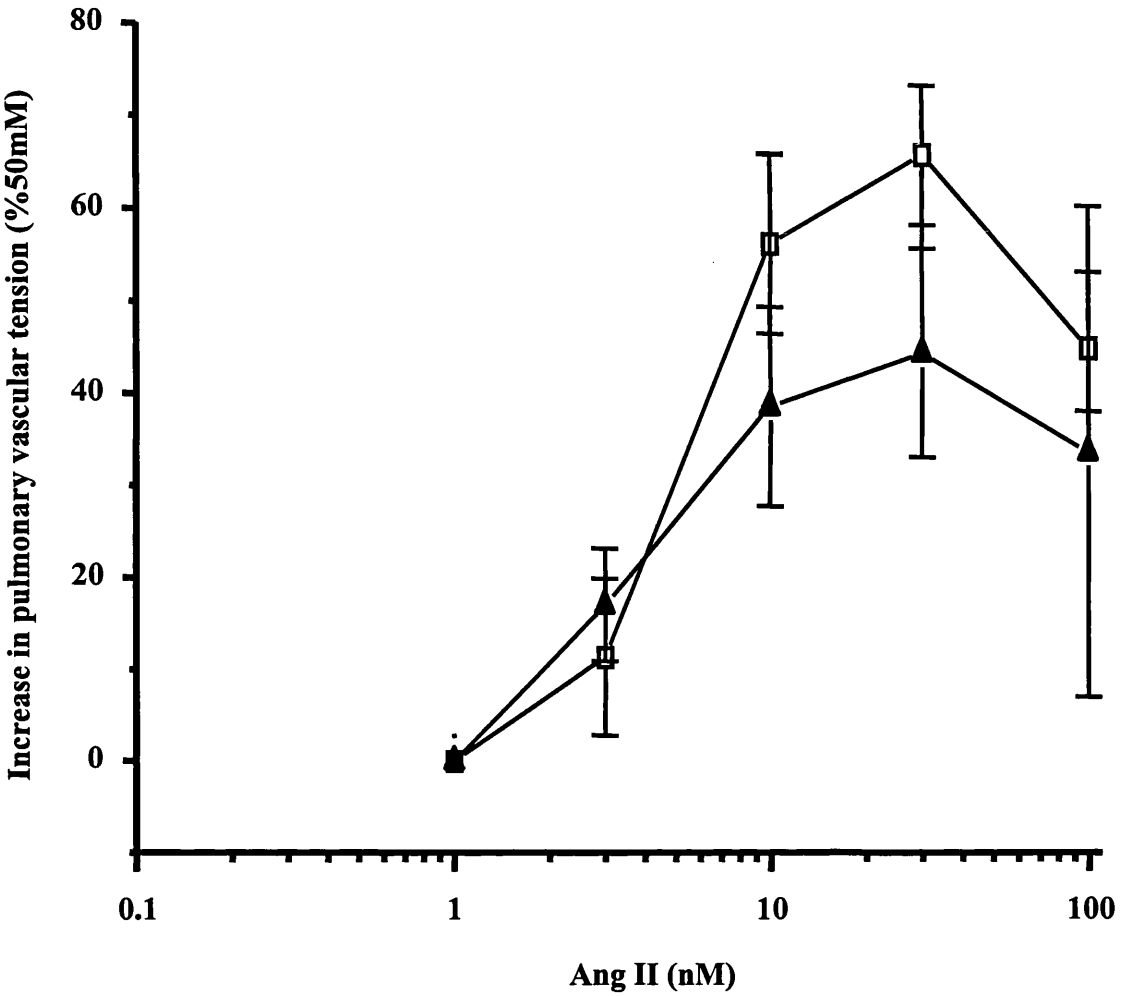

Figure 3.20: Concentration-dependent contractions to Ang II in large and small pulmonary arteries from normoxic rats.

Responses to cumulative concentrations of Ang II (1 - 100 nM) in large (□) or small (▲) pulmonary artery rings are expressed as a percentage of contraction to 50 mM KCl in the same preparation. Each point represents mean ± s. e. mean. n = 6 for the large pulmonary arteries and n = 7 for the small pulmonary arteries.

3.1.7 Effect of CH on vascular reactivity in pulmonary resistance arteries

To investigate the effect of CH in isolated pulmonary resistance arteries, vasoconstrictor responses were investigated in the fourth order branches of pulmonary artery from normoxic and CH rats.

The contractile responses to KCl and PHE were similar in pulmonary resistance arteries from the normoxic and CH rats (Figure 3.21 & 3.22). The contractile responses observed to 5-HT were potentiated in the pulmonary resistance arteries from CH rats compared to the controls (Figure 3.23). The maximal responses to 5-HT (100 μM) were 41.3 ± 9.2 % (n = 3) in the controls and 168.6 ± 33.3 % (n = 5) in CH, $P < 0.05$. Conversely, the responses to Ang II were diminished in the pulmonary resistance arteries from CH rats compared to the controls; 48.8 ± 12.3 % (n = 4) in CH rats vs. 9.8 ± 3.8 % (n = 5) in the controls to 10 nM Ang II, $P < 0.05$ (Figure 3.24).

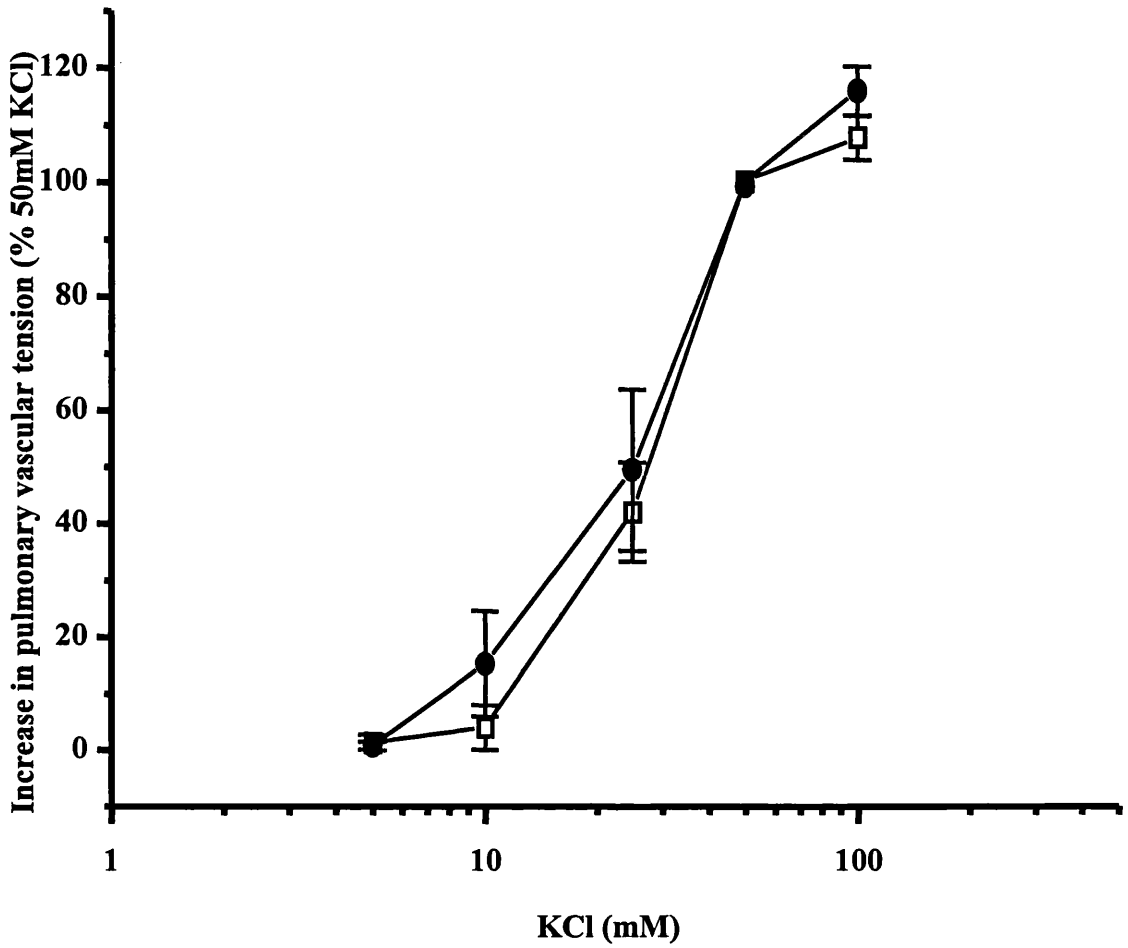

Figure 3.21: Responses to KCl in pulmonary resistance arteries from normoxic and CH rats.

Vascular responses to KCl (5 - 100 mM) in pulmonary resistance arteries from normoxic (□) or CH (●) rats are expressed as a percentage of contraction to 50 mM KCl in the same preparation. Each point represents mean ± s. e. mean. n = 3 for the controls and n = 5 for CH group.

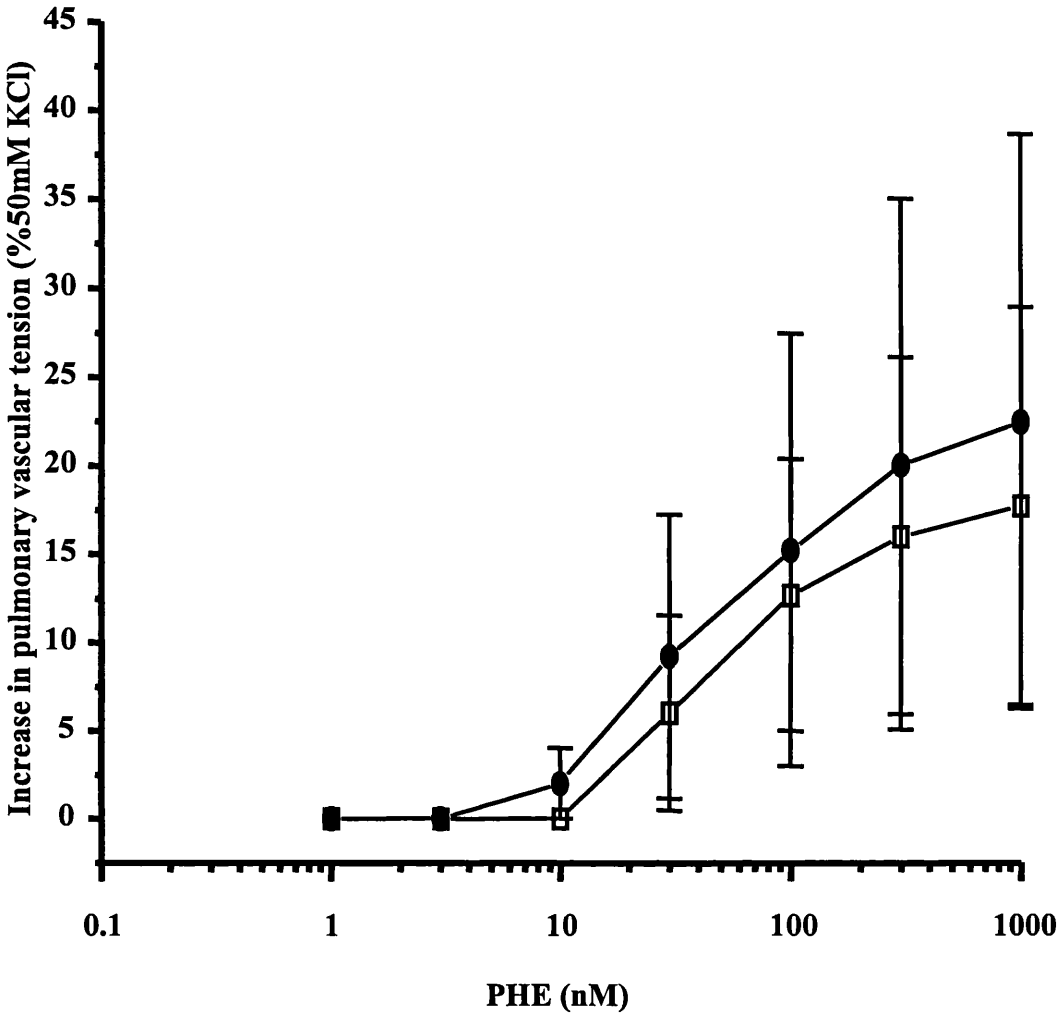

Figure 3.22: Responses to PHE in pulmonary resistance arteries from normoxic and CH rats.

Increases of tension to cumulative concentrations of PHE (1 - 1000 nM) in pulmonary resistance arteries from normoxic (□) or CH (●) rats are expressed as a percentage of contraction to 50mM KCl in the same preparation. Each point represents mean ± s. e. mean. n = 3 for the controls and n = 5 for CH group.

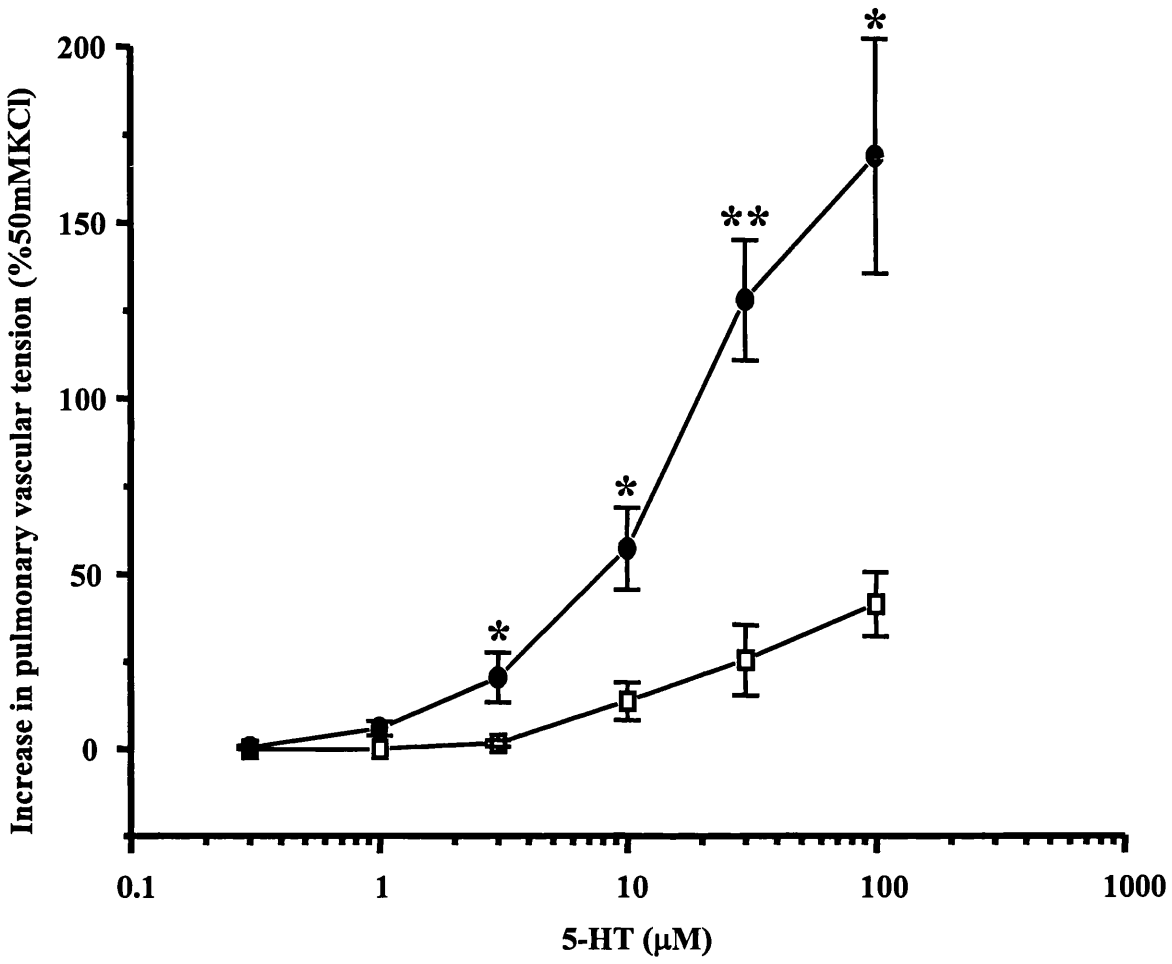

Figure 3.23: Responses to 5-HT in pulmonary resistance arteries from normoxic and hypoxic rats.

Responses to cumulative concentrations of 5-HT (0.3 - 100 μM) in pulmonary resistance arteries from normoxic (□) or CH (●) rats are expressed as a percentage of contraction to 50 mM KCl in the same preparation. Each point represents mean ± s. e. mean. *P < 0.05 vs. the controls; **P < 0.01 vs. the controls; unpaired Student's t-test. n = 3 for the controls and n = 5 for CH group.

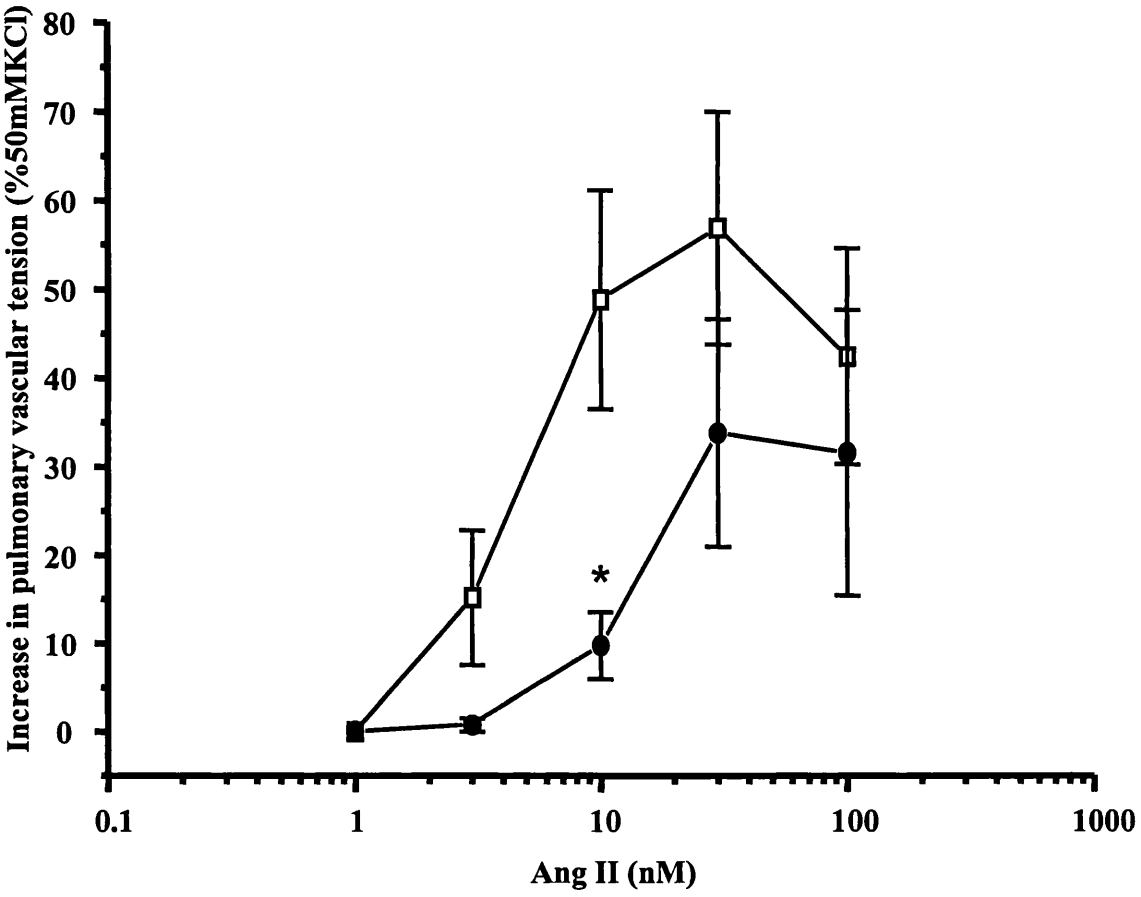

Figure 3.24: Responses to Ang II in pulmonary resistance arteries from normoxic and hypoxic rats.

Vascular responses to Ang II (1 - 100 nM) in pulmonary resistance arteries from normoxic (□) or CH (●) rats are expressed as a percentage of contraction to 50 mM KCl in the same preparation. Each point represents mean ± s. e. mean. *P < 0.05 vs. the controls; unpaired Student's t-test. n = 5 for the controls and n = 4 for CH group.

3.2 Relationship between pulmonary vascular hyper-reactivity and pulmonary vascular remodelling in CH

3.2.1 Preliminary experiments for time-dependent study of vasoconstrictor responses in isolated lungs

Initial experiments were carried out to ascertain the course of pulmonary hyper-reactivity during hypoxia and the recovery period. Three agonists (PHE, Ang II and KCl) were tested in this study.

The vasoconstrictor responses to PHE in isolated perfused lungs after 3 days and 7 days hypoxia were similar to the controls. PHE-induced vasoconstrictor responses started to increase after 14 days hypoxia and were significantly higher after 21 days hypoxia than the controls. For example, 10 nmoles PHE induced increases of PPP of 15.4 ± 3.0 mmHg ($n = 5$) after 21 days hypoxia and 2.7 ± 0.3 mmHg ($n = 7$) in the controls, $P < 0.05$. The vasoconstrictor responses to PHE declined gradually after 3 days and 7 days recovery. After 14 days recovery, PHE responses had returned to the control levels. PPP increases induced by 10 nmoles PHE were 3.6 ± 0.6 mmHg ($n = 6$) after 14 days recovery (Figure 3.25 & 3.26).

Hypoxia gradually potentiated the vasoconstrictor responses to Ang II as the duration of hypoxia increased. After 7 days hypoxia, the PPP increases induced by Ang II were significantly different from the controls. For example, 100 pmoles Ang II induced the

increases of PPP as 6.8 ± 1.1 mmHg in the controls (n = 4) and 17.3 ± 3.7 mmHg in at 21 days hypoxia (n = 5), $P < 0.05$. During the recovery from 21 days hypoxia, the vasoconstrictor responses to Ang II were still higher than in normoxia. Increases in PPP induced by Ang II were still similar to 21 days hypoxia, even after 21 days recovery (12.0 ± 1.3 mmHg to 100 pmoles Ang II, n = 6) (Figure 3.27 & 3.28).

The vasoconstrictor responses to KCl in the isolated lungs after 3 days and 7 days hypoxia were similar to the controls. After 14 days hypoxia, KCl responses started to increase. The significant increases appeared after 21 days hypoxia. Responses did not reverse to the control level even after 21 days recovery. For example, PPP increases to 200 μmoles KCl were 8.5 ± 1.2 mmHg (n = 6) in the controls, 18.7 ± 3.8 mmHg (n = 5) after 21 days hypoxia and 14.2 ± 1.0 mmHg (n = 6) after 21 days recovery (Figure 3.29 & 3.30).

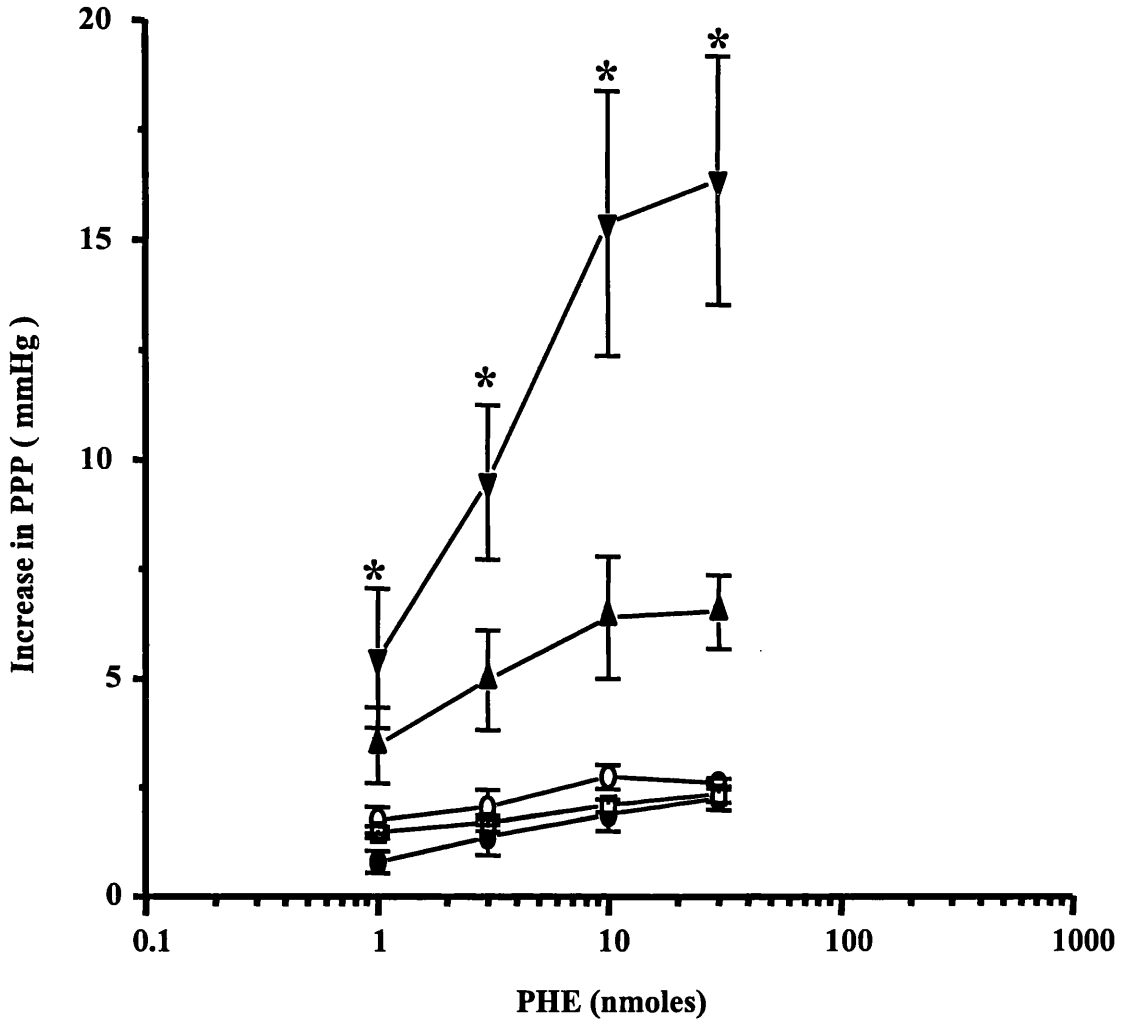

Figure 3.25: Vasoconstrictor responses to PHE in isolated perfused lungs from normoxic rats and those exposed to different periods of hypoxia.

Data are presented as increases of PPP (mmHg) to bolus injections of PHE (1-30 nmoles) into the lungs from normoxic (○), 3 days hypoxic (●), 7 days hypoxic (□), 14 days hypoxic (▲) and 21 days hypoxic (▼) animals. Each point represents mean ± s. e. mean. *P < 0.05 vs. the normoxic group; one-way ANOVA with Dunnett's test. n = 5 - 7 for each group.

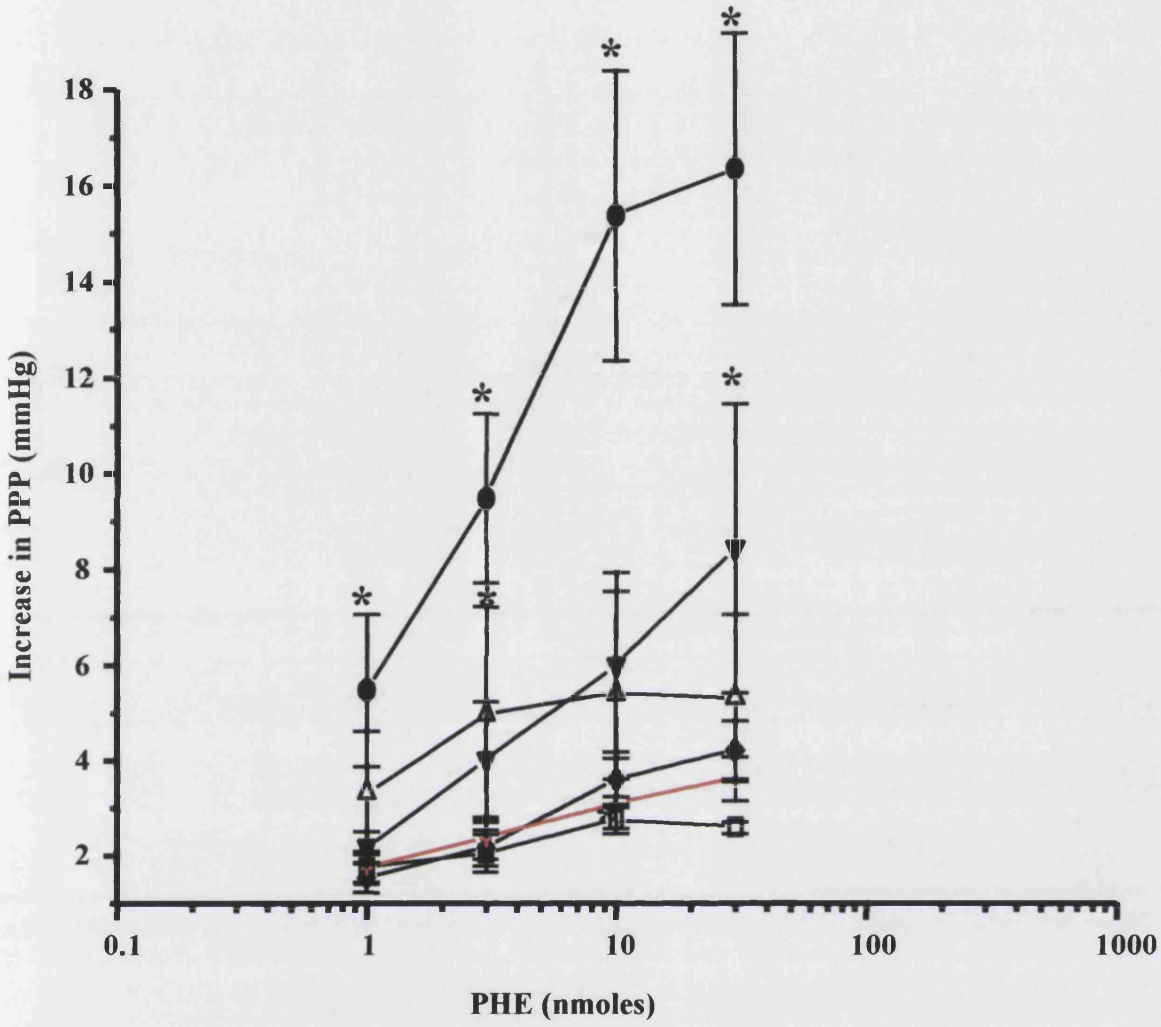

Figure 3.26: Vasoconstrictor responses to PHE in isolated perfused lungs from normoxic rats, 21 days hypoxic rats and those exposed to different periods of recovery.

Data are presented as increases of PPP (mmHg) to bolus injections of PHE (1-30 nmoles) into the lungs from normoxic (□), 21 days hypoxic (●), 3 days recovery (△), 7 days recovery (▼), 14 days recovery (◆) and 21 days recovery (+) animals. Each point represents mean ± s. e. mean. *P < 0.05 vs. the normoxic group; one-way ANOVA with Dunnett's test. n = 5 - 6 for each group. Re-use data from normoxic and 21 days hypoxic rats as negative and positive controls, respectively.

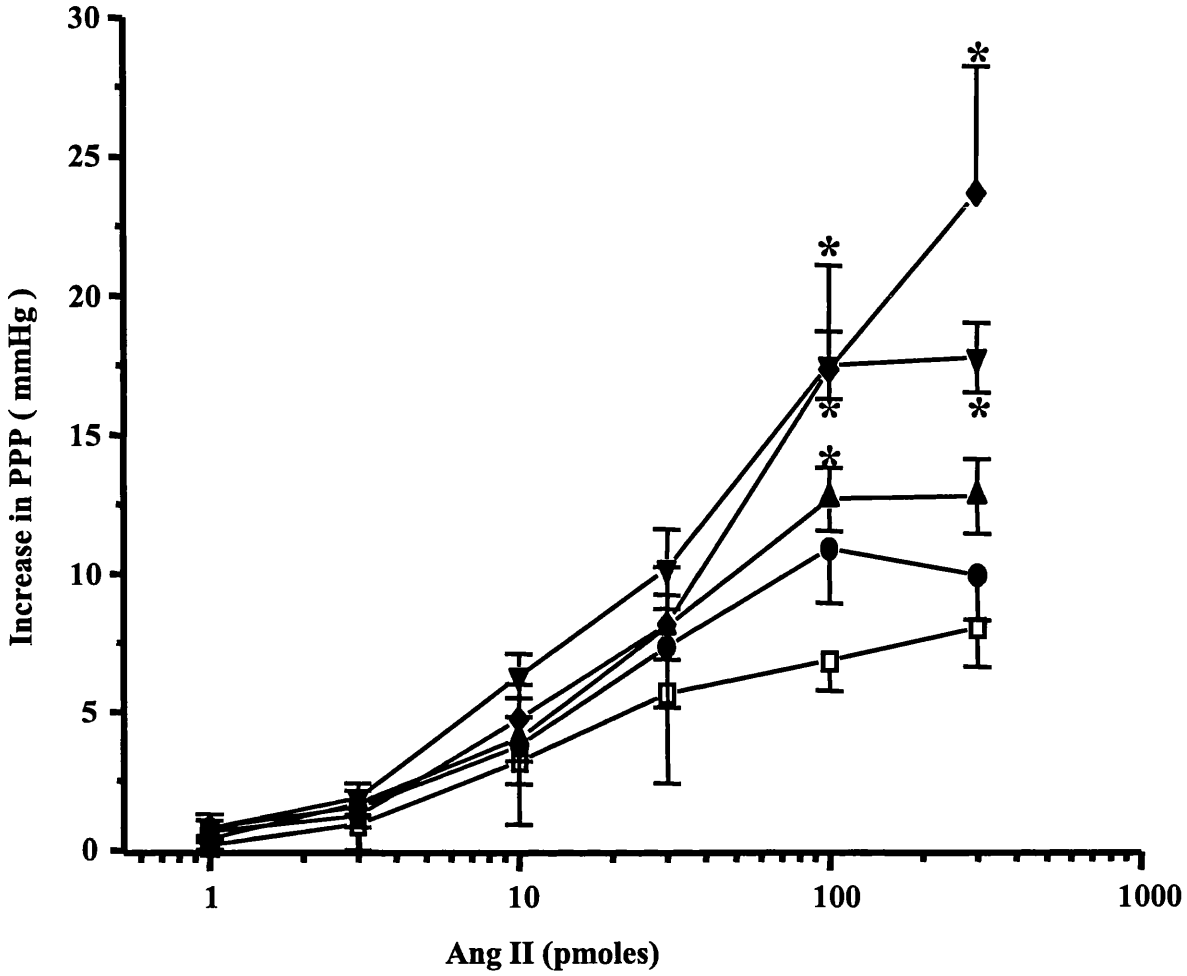

Figure 3.27: Vasoconstrictor responses to Ang II in isolated perfused lungs from normoxic rats and those exposed to different periods of hypoxia.

Data are presented as increases of PPP (mmHg) to bolus injections of Ang II (1-300 pmoles) into the lungs from normoxic (□), 3 days hypoxic (●), 7 days hypoxic (▲), 14 days hypoxic (▼) and 21 days hypoxic (◆) animals. Each point represents mean ± s. e. mean. *P < 0.05 vs. the normoxic group; one-way ANOVA with Dunnett's test. n = 4 - 5 for each group.

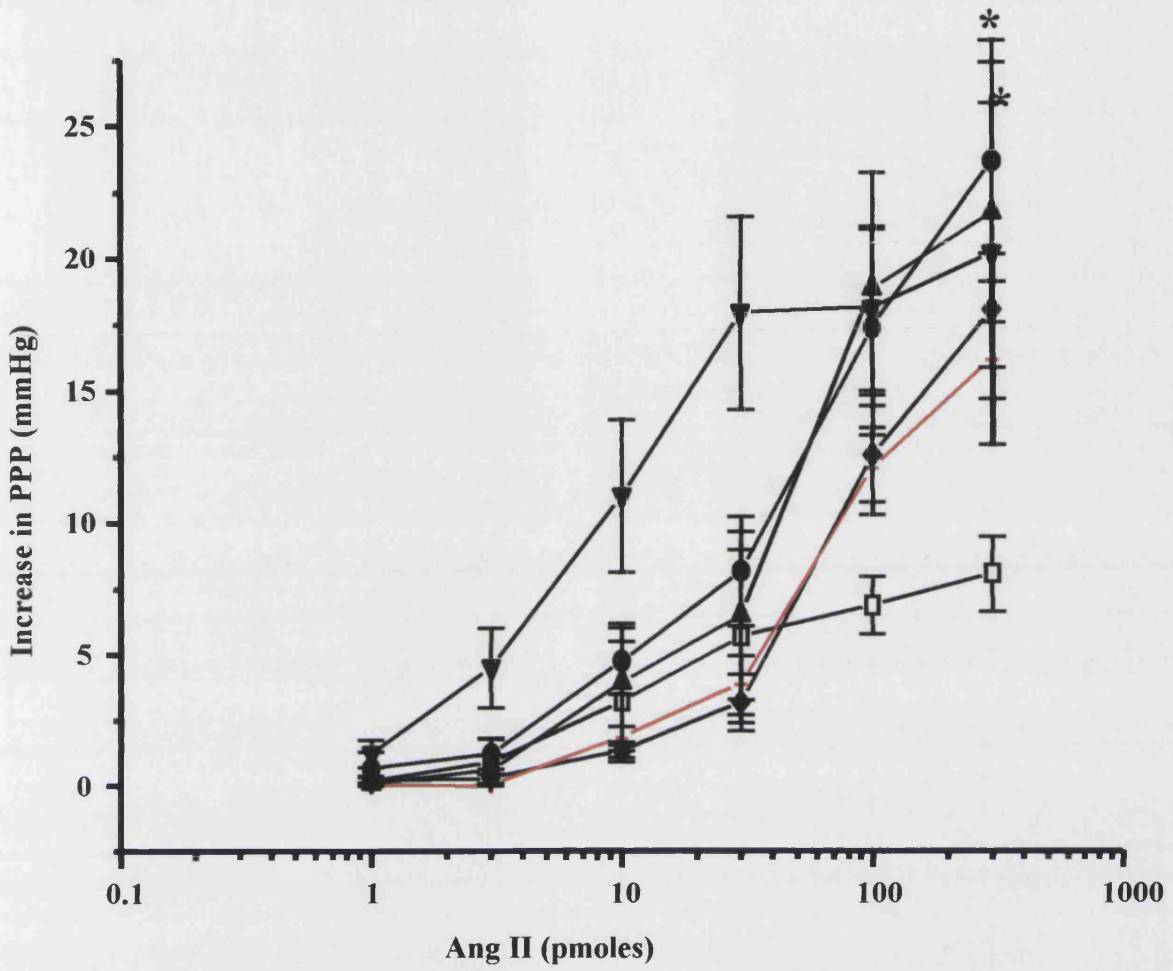

Figure 3.28: Vasoconstrictor responses to Ang II in isolated perfused lungs from normoxic rats, 21 days hypoxic rats and those exposed to different periods of recovery.

Data are presented as increases of PPP (mmHg) to bolus injections of Ang II (1 - 300 pmoles) into the lungs from normoxic (□), 21 days hypoxic (●), 3 days recovery (▲), 7 days recovery (▼), 14 days recovery (◆) and 21 days recovery (+) animals. Each point represents mean ± s. e. mean. *P < 0.05 21 days hypoxic group and 3 days recovery group vs. the normoxic group; one-way ANOVA with Dunnett's test. n = 5 - 6 for each group. Re-use data from normoxic and 21 days hypoxic rats as negative and positive controls, respectively.

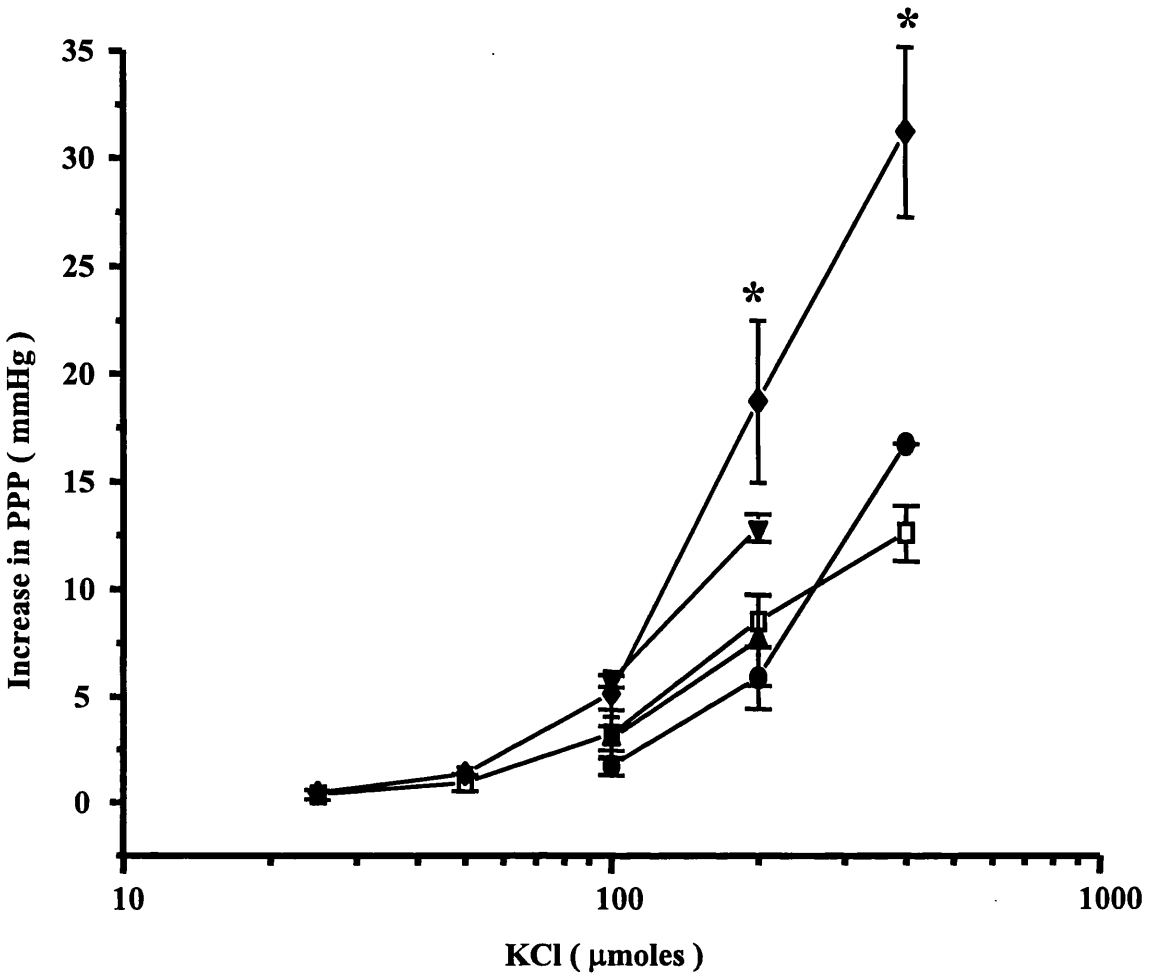

Figure 3.29: Vasoconstrictor responses to KCl in isolated perfused lungs from normoxic rats and those exposed to different periods of hypoxia.

Data are presented as increases of PPP (mmHg) to bolus injections of KCl (25 - 400 μmoles) into the lungs from normoxic (□), 3 days hypoxic (●), 7 days hypoxic (▲), 14 days hypoxic (▼) and 21 days hypoxic (◆) animals. Each point represents mean ± s. e. mean. *P < 0.05 vs. the normoxic group; one-way ANOVA with Dunnett's test. n = 4 - 6 for each group.

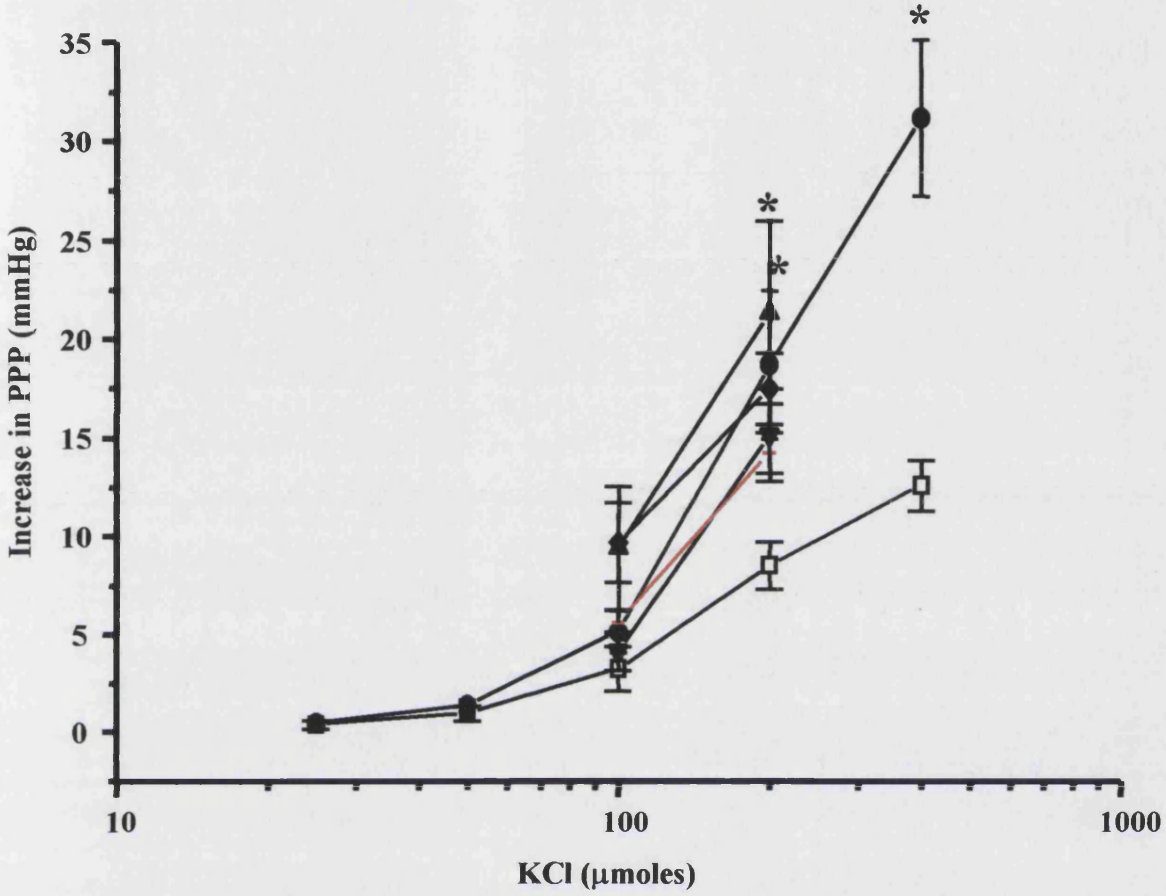

Figure 3.30: Vasoconstrictor responses to KCl in isolated perfused lungs from normoxic rats, 21 days hypoxic rats and those exposed to different periods of recovery.

Data are presented as increases of PPP (mmHg) to bolus injections of KCl (25 - 400 μmoles) into the lungs from normoxic (□), 21 days hypoxic (●), 3 days recovery (▲), 7 days recovery (▼), 14 days recovery (◆) and 21 days recovery (+) animals. Each point represents mean ± s. e. mean. *P < 0.05 21 days hypoxia or 3 days recovery vs. the normoxic group; one-way ANOVA with Dunnett's test. n = 5 - 6 for each group. Re-use data from normoxic and 21 days hypoxic rats as negative and positive controls, respectively.

3.2.2 Indices of hypoxic pulmonary hypertension after 21 days hypoxia and 21 days recovery

As determined from the preliminary experiments, 21 days hypoxia and 21 days recovery rats were chosen for comparison of pulmonary vascular hyper-reactivity and pulmonary remodelling in CH.

Body weights were 300 ± 9.5g (n = 5) in the rats after 21 days hypoxia, 285.5 ± 6.1g (n = 11) in the age-matched controls; 408.6 ± 13g (n = 5) in the recovery rats and 419.2 ± 9.6g (n = 12) in the age-matched controls.

Figure 3.31 shows the haematocrits during normoxia, after 21 days hypoxia and 21 days recovery. 21 days hypoxia increased haematocrit significantly from 46.5 ± 0.6% (n = 4) in the controls to 57 ± 0% (n = 4) after 21 days hypoxia ($P < 0.05$). After 21 days recovery, haematocrit returned to 49 ± 1.4%, similar to the normoxic level ($P > 0.05$, n = 4).

Basal PPP increased from 6.3 ± 0.9 mmHg (n = 5) in the controls to 10.5 ± 0.2 mmHg (n = 5) after 21 days hypoxia ($P < 0.05$). After 21 days recovery, the basal PPP returned to a level (6.4 ± 0.5mmHg, n = 6) similar to the controls, $P > 0.05$ (Figure 3.32).

21 days hypoxia induced significant right ventricular hypertrophy as measured by the ratio of right ventricular weight to total ventricular weight; 0.205 ± 0.013 in the controls

vs. 0.338 ± 0.010 after 21 days hypoxia, $P < 0.05$. The ratio was still significantly elevated afer 21 days recovery (0.270 ± 0.019) as compared to the control group, $P < 0.05$, $n = 5 - 6$ (Figure 3.33).

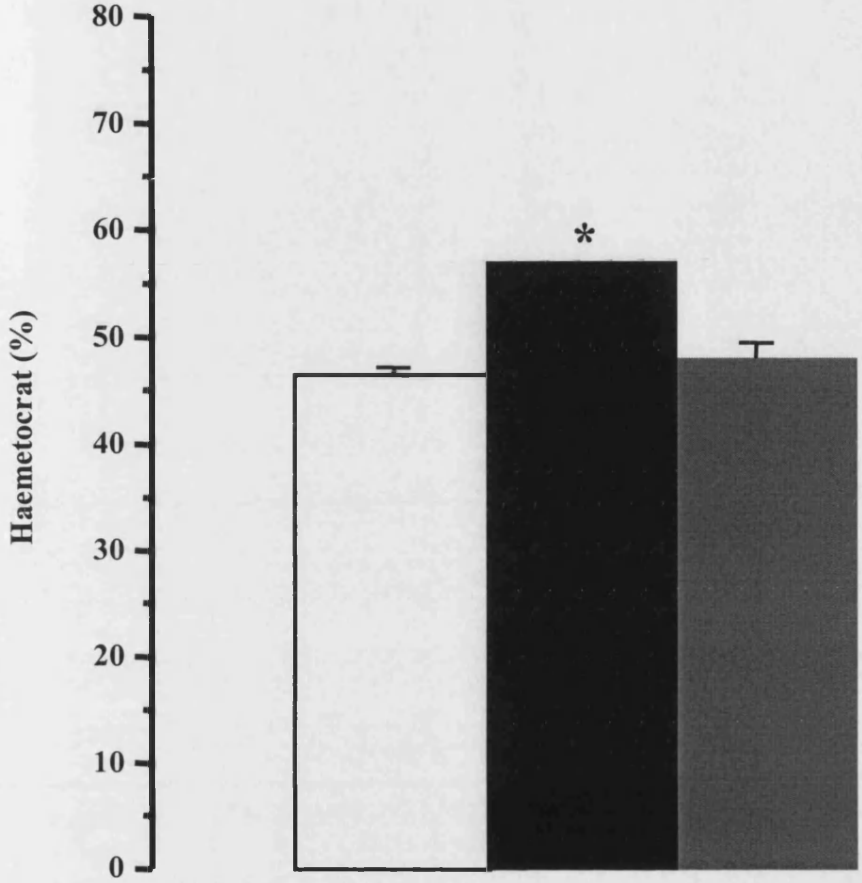

Figure 3.31: Haematocrit in normoxic, 21 days hypoxic and 21 days recovery rats.

Blood samples were collected from the normoxic ( ), 21 days hypoxic ( ) and 21 days recovery ( ) rats. Each column represents mean ± s. e. mean, *P < 0.05 vs. the normoxic group; one-way ANOVA with Dunnett's test. n = 4 for each group.

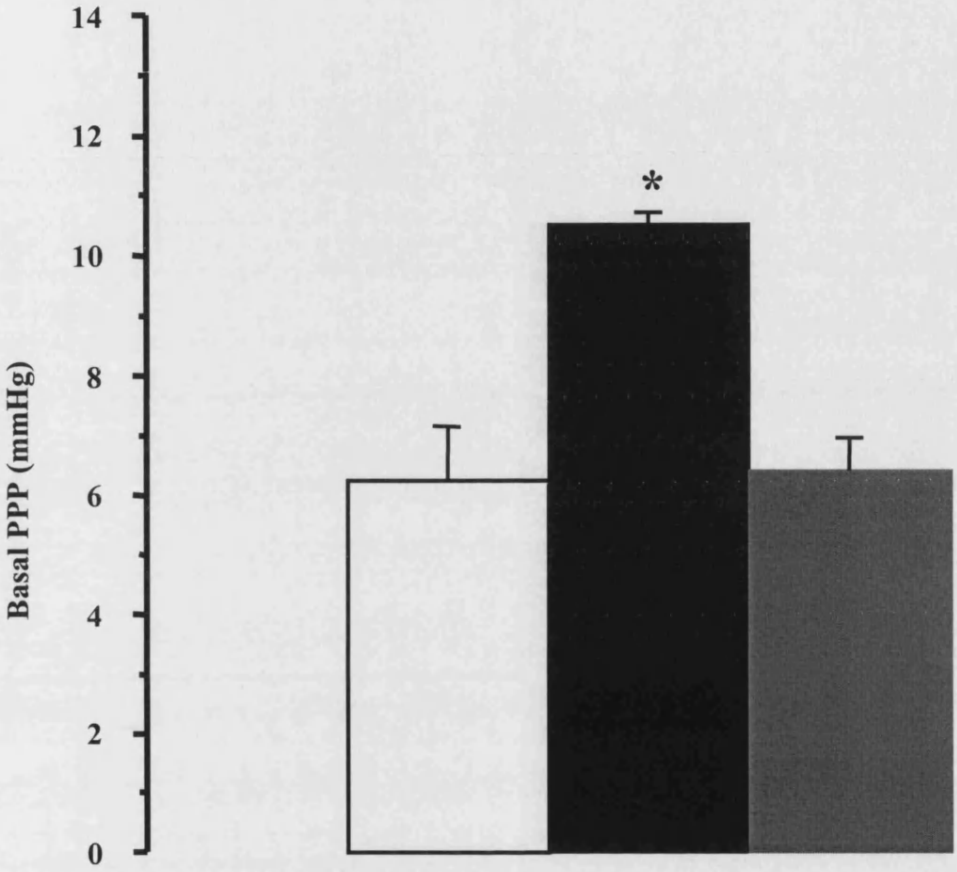

Figure 3.32: Basal PPP in isolated perfused lungs from normoxic, 21 days hypoxic and 21 days recovery rats.

Basal PPP in 21 days hypoxic rats (■) was significantly higher than in the control group (□). After 21 days recovery, basal PPP was returned to a level (■) similar to the controls. Each column represents mean ± s. e. mean. *P < 0.05 vs. the normoxic group; one-way ANOVA with Dunnett's test. n = 5 - 6 for each group.

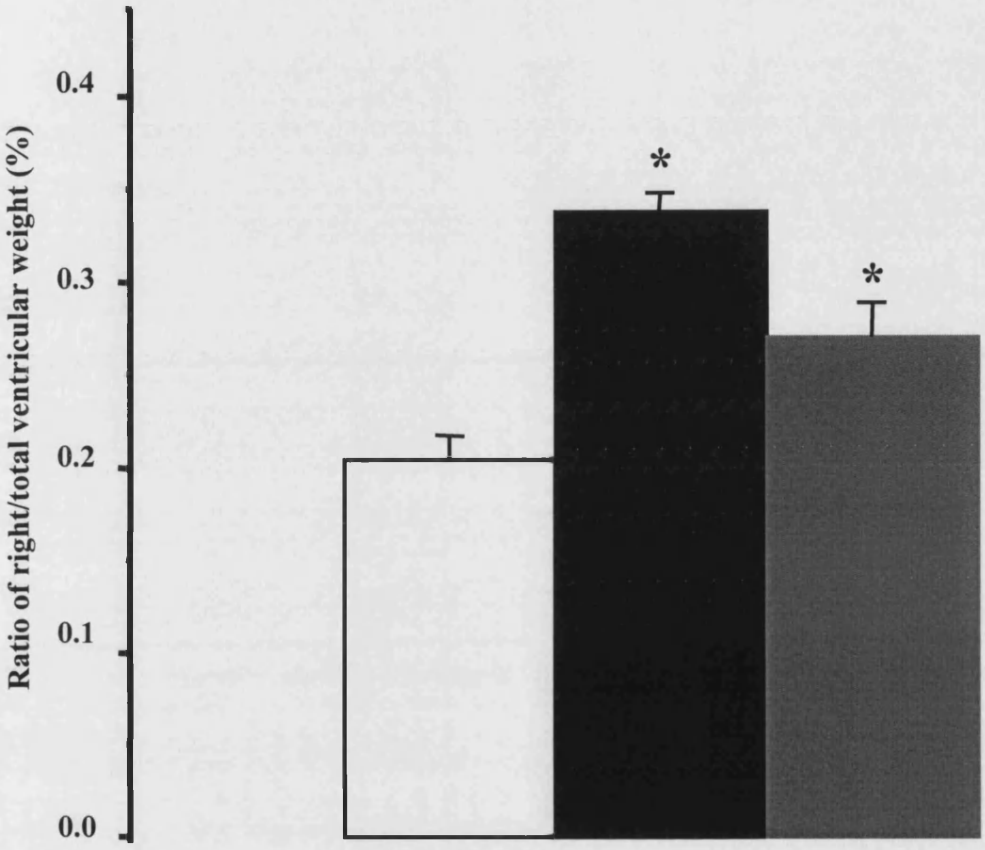

Figure 3.33: Index of right ventricular hypertrophy from normoxic, 21 days hypoxic and 21 days recovery rats.

The ratio of right ventricular weight to total ventricular weight was taken as index of right ventricular hypertrophy from the normoxic ( ), 21 days hypoxic ( ) and 21 days recovery ( ) rats. Each column represents mean ± s. e. mean. *P < 0.05 vs. the normoxic group; one-way ANOVA with Dunnett's test. n = 5 - 6 for each group.

3.2.3 Effect of CH on structural changes of pulmonary vascular wall thickness

Figure 3.34 shows the typical micrographs of pulmonary arteries, which were similar size vessels from normoxic, 21 days hypoxic and 21 days recovery rats. The medial wall thickness of pulmonary arteries significantly increased in 21 days hypoxic rats. There are more vascular smooth muscle cells and elastic laminae in the pulmonary arterial wall after 21 days hypoxia, compared to the control; and the internal elastic lamina of the pulmonary artery is much clearer in 21 days hypoxic lungs than in the normoxic vessel. The hyperplasia of the vascular smooth muscle cells and increased elastic laminae were still apparent after 21 days recovery. 21 days hypoxia increased the medial wall thickness at all levels of pulmonary artery (Table 3.2). The pulmonary medial wall thickness was significantly increased from 17.5 ± 1% (n = 84) in the controls to 21.8 ± 0.9% (n = 106) after 21 days hypoxia, $P < 0.01$. After 21 days recovery, the pulmonary medial wall thickness (25.7 ± 1%, n = 89) was still higher than the age-matched control group (18.4 ± 0.7%, n = 114), $P < 0.001$ (Figure 3.35).

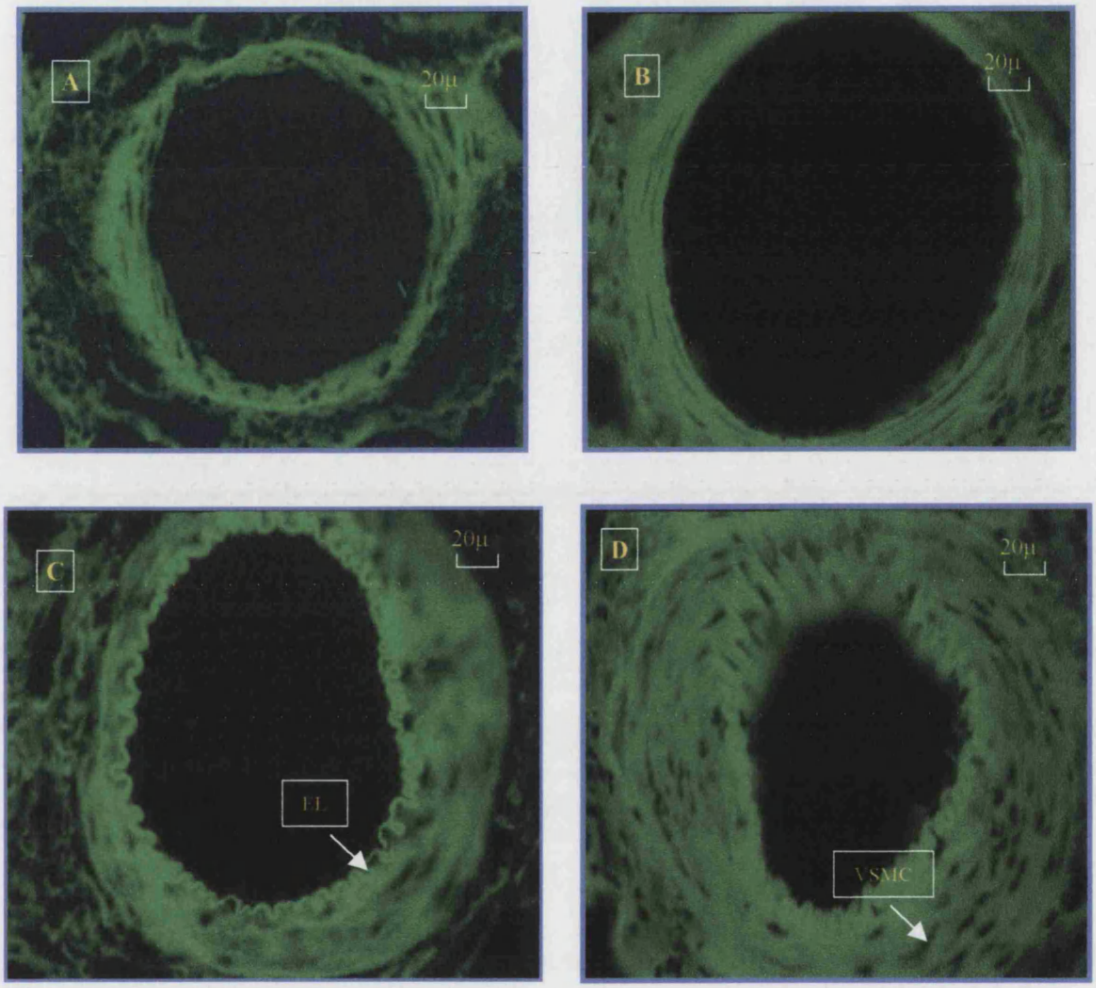

Figure 3.34: Pulmonary vascular remodellings after 21 days hypoxia and 21 days recovery.

Representative fluorescence micrographs (× 400 magnification) show that in the age-matched control lungs (**A**), the pulmonary arterial walls are thin and 21 days hypoxia (**C**) induces pulmonary vascular hyperplasia including proliferation of vascular smooth muscle cells (VSMC) and increase of elastic lamina (EL). During 21 days recovery (**D**), pulmonary vascular hyperplasia is still apparent compared to the age-matched controls (**B**).

Table 3.2 Measurement of medial wall thickness and diameter of pulmonary arteries

	Control (10W)	21 days hypoxia	Control (13W)	Recovery
Total vessels				
No. of rats	4	5	5	4
No. of vessels	84	106	114	89
Ratio of wall thickness (%)	17.5 ± 1	21.8 ± 0.9**	18.4 ± 0.7	25.7 ± 1***
Vessel diameter (μm)	143.7 ± 6.5	193 ± 10***	176.9 ± 9.1	151 ± 13*
Diameter range (μm)	55 - 287	45 - 492	37 - 622	37 - 815
Vessel diameter < 150 μm				
No. of vessels	50	45	61	56
Ratio of wall thickness (%)	17.6 ± 1	24.2 ± 1.6***	20.1 ±1.0	27.6 ± 1.7***
Vessel diameter (μm)	105.7 ± 3.8	99 ± 3.4*	108.6 ±3.4	82 ± 4.1***
Diameter range (μm)	55 - 147	45 - 147	37 - 149	37 - 147
Vessel diameter > 150 μm				
No. of vessels	34	61	53	33
Ratio of wall thickness (%)	13.8 ±1.2	20 ± 0.9***	16.5 ± 0.9	22.5 ±1.8**
Vessel diameter (μm)	199.6 ± 8.6	261.9 ± 11***	256 ± 12	268 ± 23*
Diameter range (μm)	155 - 287	160 - 492	150 - 622	151 - 815

The vessel number on each slide is 6-10. The age-matched controls to 21 day hypoxia were 10 weeks rats; whereas the age-matched controls to 21 days recovery were 13 weeks rats. Data for the ratio of wall thickness and vessel diameter are mean ± s. e. mean. *$P < 0.05$ vs. the age-matched controls; **$P < 0.01$ vs. the age-matched controls; ***$P < 0.001$ vs. the age-matched controls; unpaired Student's t-test.

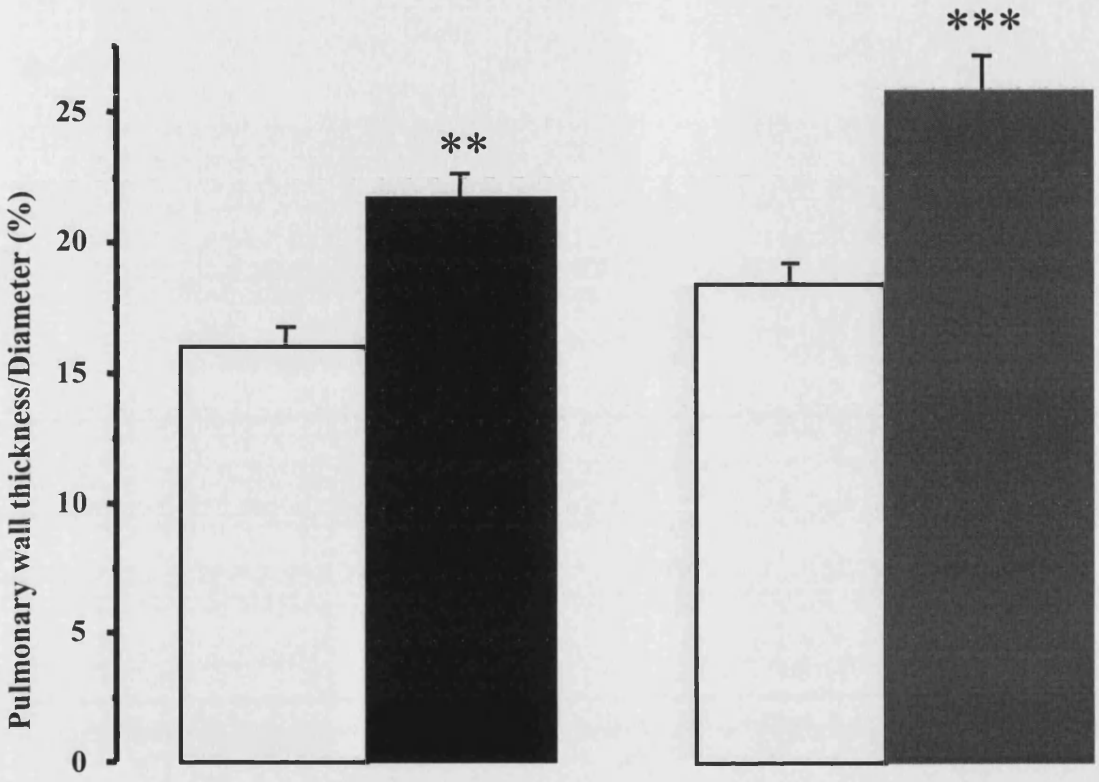

Figure 3.35: Pulmonary medial wall thickness in normoxic, 21 days hypoxic and 21 days recovery rats.

Ratio of pulmonary medial wall thickness was measured from the normoxic rats ( ), age-matched to 21 days hypoxic ( ) or 21 days recovery ( ) rats. Each column represents mean ± s. e. mean. **P < 0.01 vs. the age-matched controls; ***P < 0.001 vs. the age-matched controls; unpaired Student's t-test. n = 106 for 21 days hypoxia group and n = 84 for its age-matched controls. n = 89 for 21 days recovery group and n = 114 for its age-matched controls. Data were shown in Table 3.2.

3.3 ET-1 sensitization in pulmonary vasculature

3.3.1 Determination of the sub-threshold concentration of ET-1 infusion in isolated lungs.

Initial experiments were carried out to determine a sub-threshold concentration of ET-1 in the isolated perfused lungs. Figure 3.36 shows the concentration-vasoconstriction response curve produced by ET-1. 0.3 nM and 1nM ET-1 did not change PPP. PPP started to increase from infusion of 3nM ET-1, which induced a significant increase (10.9 ± 0.6 mmHg) when compared with the control group (8.5 ± 0.6 mmHg), $P < 0.05$, $n = 4$.

Another fact, which can affect ET-1 sensitization to vasoconstrictors, is the duration of ET-1 infusion. Figure 3.37 shows that from 30 min infusion of ET-1 (1 nM), ET-1 significantly potentiated the vasoconstrictor responses to Ang II (30 pmoles) compared to the controls; 3.4 ± 0.5 mmHg for 30 min infusion of ET-1 vs. 1.6 ± 0.3 mmHg for 0 min infusion of ET-1, $n = 7$, $P < 0.05$.

Thus, in all subsequent experiments 1nM ET-1 had being infused into the pulmonary circulation for 30 min period before agonist additions.

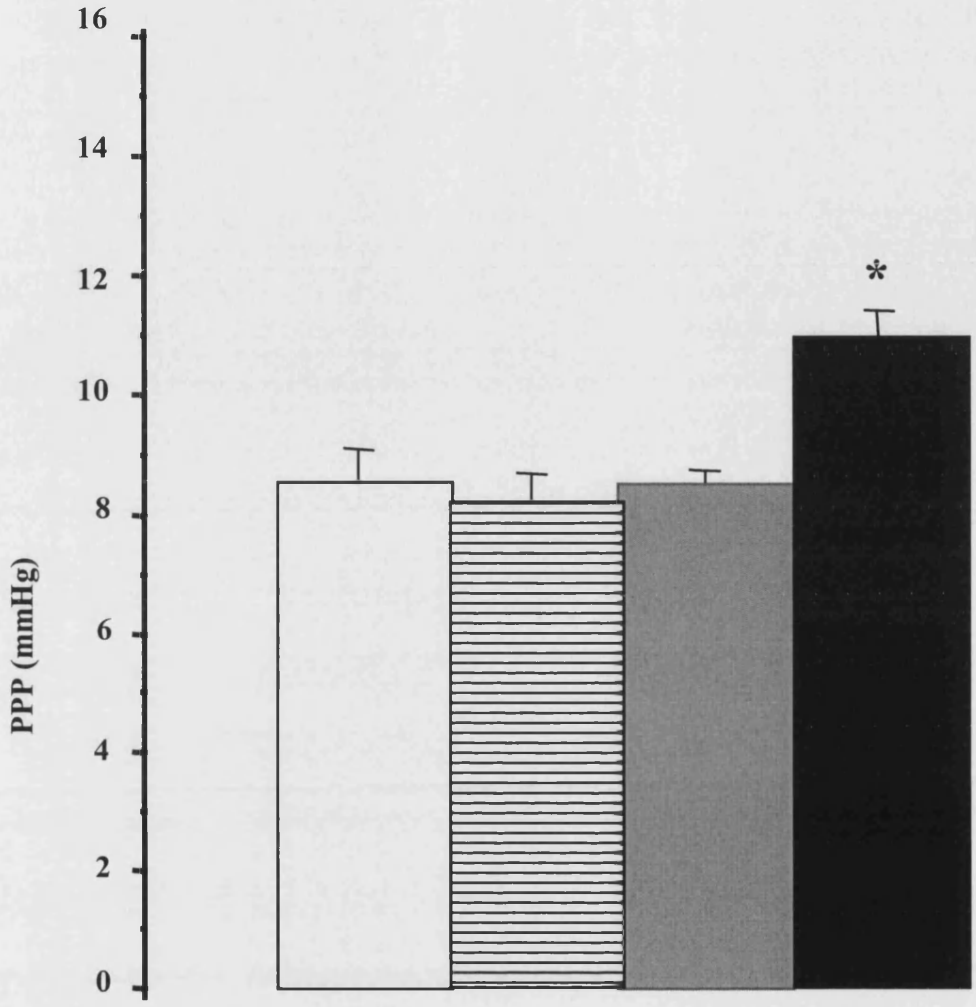

Figure 3.36: Effect of ET-1 on basal PPP in the isolated perfused lungs.

Isolated perfused lungs were perfused with Krebs' solution as the controls ( ) or with infusion of 0.3 nM ET-1 ( ), 1 nM ET-1 ( ) or 3 nM ET-1 ( ) for 15 min. Each column represents mean ± s. e. mean. *P < 0.05 vs. the controls; one way ANOVA followed by Dunnett's test. n = 4 for each group.

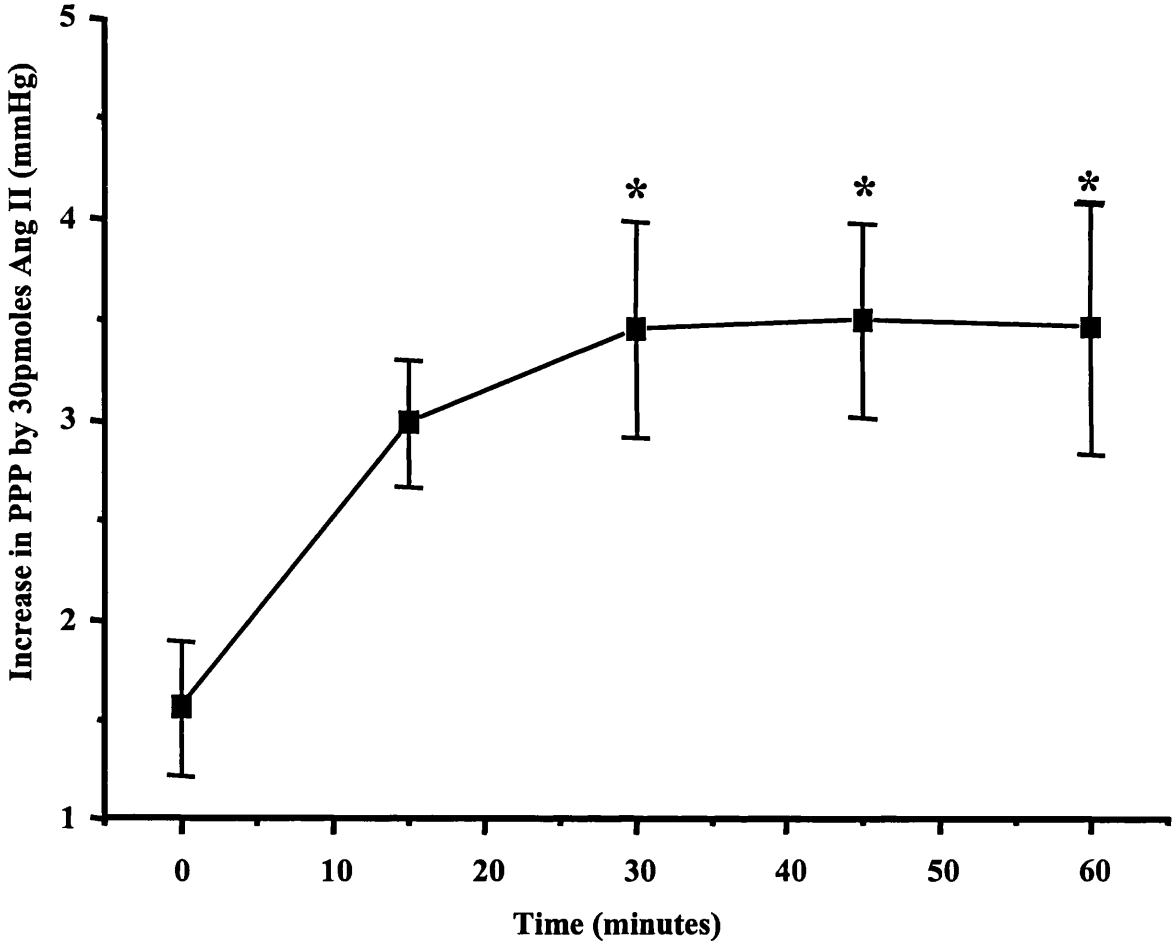

Figure 3.37: Effect of varying infusion times of ET-1 on Ang II-induced vasoconstrictor responses in isolated perfused lungs.

Graph shows effects of infusing ET-1 (1 nM) over various time points on responses to Ang II (30 pmoles), compared to the controls (no ET-1 infusion, time 0). Each point represents mean ± s. e. mean. * $P < 0.05$ vs. 0 min infusion of ET-1; one way ANOVA followed by Dunnett's test. n = 7 for each point.

3.3.2 Effect of ET-1 on responses to Ang II in isolated lungs

Dose-responses to Ang II in isolated perfused lungs from normoxic rats were recorded before and after infusing the sub-threshold vasoconstrictor concentration of ET-1 (1 nM) for 30 minutes. The dose-response curve of Ang II was shifted to the left when infusing ET-1, compared with the controls (Figure 3.38). Responses to 10 pmoles Ang II were increased by ET-1 from 0.99 ± 0.17 mmHg in controls to 2.57 ± 0.42 mmHg with ET-1 ($n = 8$, $p < 0.05$).

The antagonists were infused for 15 min and the duration of ET-1 infusion for sensitizing the pulmonary vasculature was still 30 min before bolus-injection of agonists. PD156707, a selective ET_A receptor antagonist, and BQ788, a selective ET_B receptor antagonist, both inhibited the potentiation of the vasoconstrictor responses to Ang II with ET-1 in the isolated perfused lungs from normoxic rats (Figure 3.39 & 3.40). The vasoconstrictor responses to 10 pmoles Ang II with ET-1 were reduced by PD156707 (5 μM) from 2.57 ± 0.42 mmHg in the ET-1 controls to 0.95 ± 0.24 mmHg with ET-1 plus PD156707 ($n = 4 - 8$, $P < 0.01$). BQ788 (5 μM) shifted the vasoconstrictor responses to 10 pmoles Ang II from 2.57 ± 0.42 mmHg in ET-1 controls to 0.93 ± 0.33mmHg with ET-1 plus BQ788 ($n = 5 - 8$, $P < 0.05$).

Neither PD156707 (5 μM) nor BQ788 (5 μM) had any effect on the vasoconstrictor responses to Ang II alone in the isolated perfused lungs from normoxic rats.

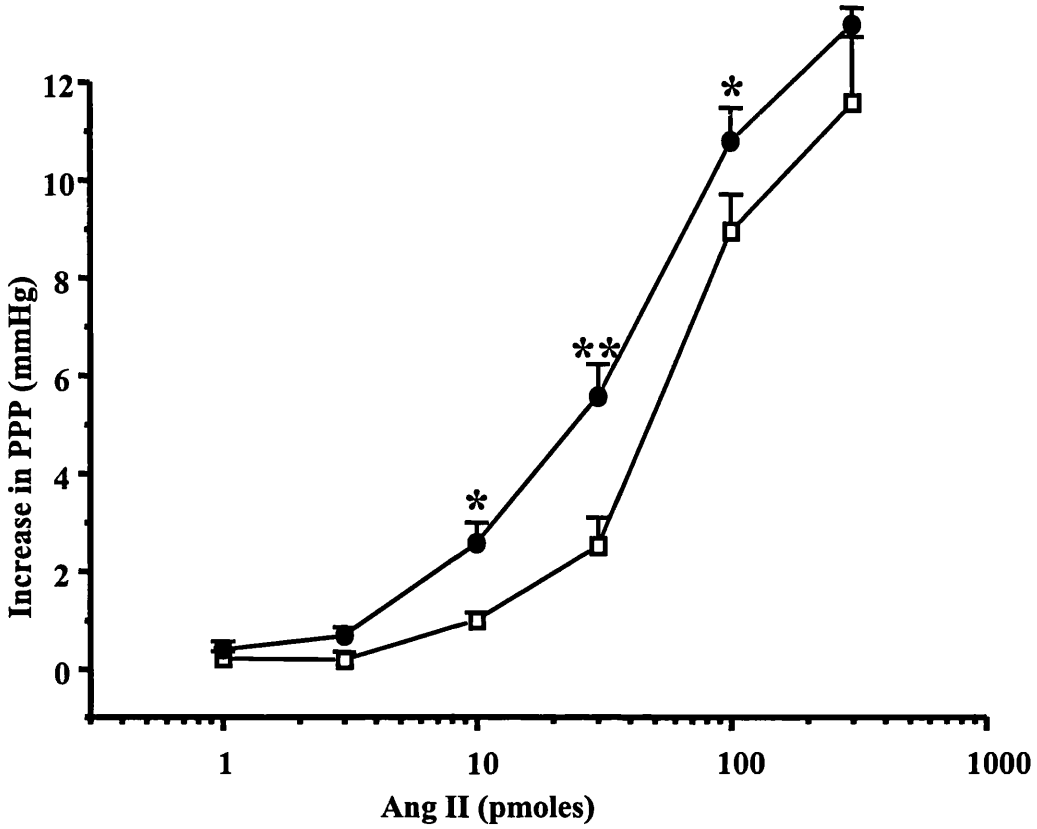

Figure 3.38: Effect of ET-1 on vasoconstrictor responses to Ang II in normoxic isolated perfused lungs.

Data are presented as increases of PPP (mmHg) to bolus injections of Ang II (1 - 300 pmoles) into the lungs in the controls (□) or with ET-1 (1nM) (●). Each point represents mean ± s. e. mean. *P < 0.05 vs. the controls; **P < 0.01 vs. the controls; unpaired Student's t-test. n = 8 for each group.

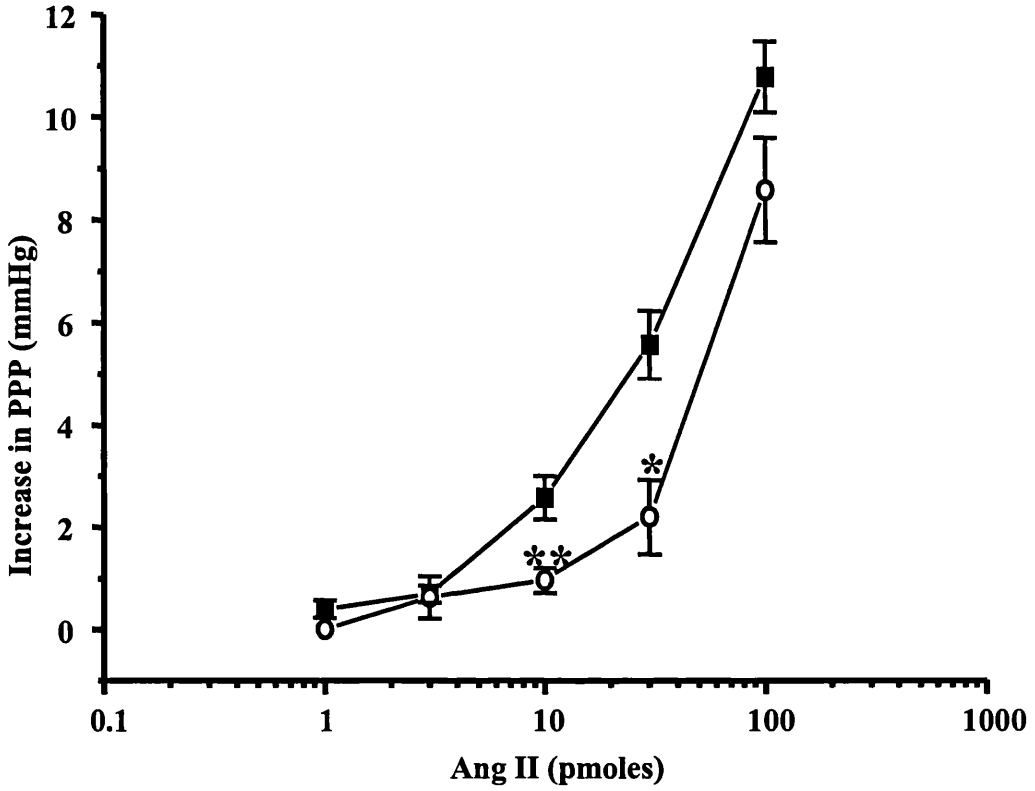

Figure 3.39: Effect of a selective ET_A receptor antagonist, PD156707 on the vasoconstrictor responses to Ang II with ET-1 in normoxic isolated perfused lungs.

Data are presented as increases of PPP (mmHg) to bolus injections of Ang II (1 - 100 pmoles) into the lungs with ET-1 alone (1nM) (■) or with ET-1 plus PD156707 (5 μM) (O). Each point represents mean ± s. e. mean. *P < 0.05 vs. ET-1 controls; **P < 0.01 vs. ET-1 controls; unpaired Student's t-test. n = 8 for ET-1 controls and n = 4 for PD156707 treated group.

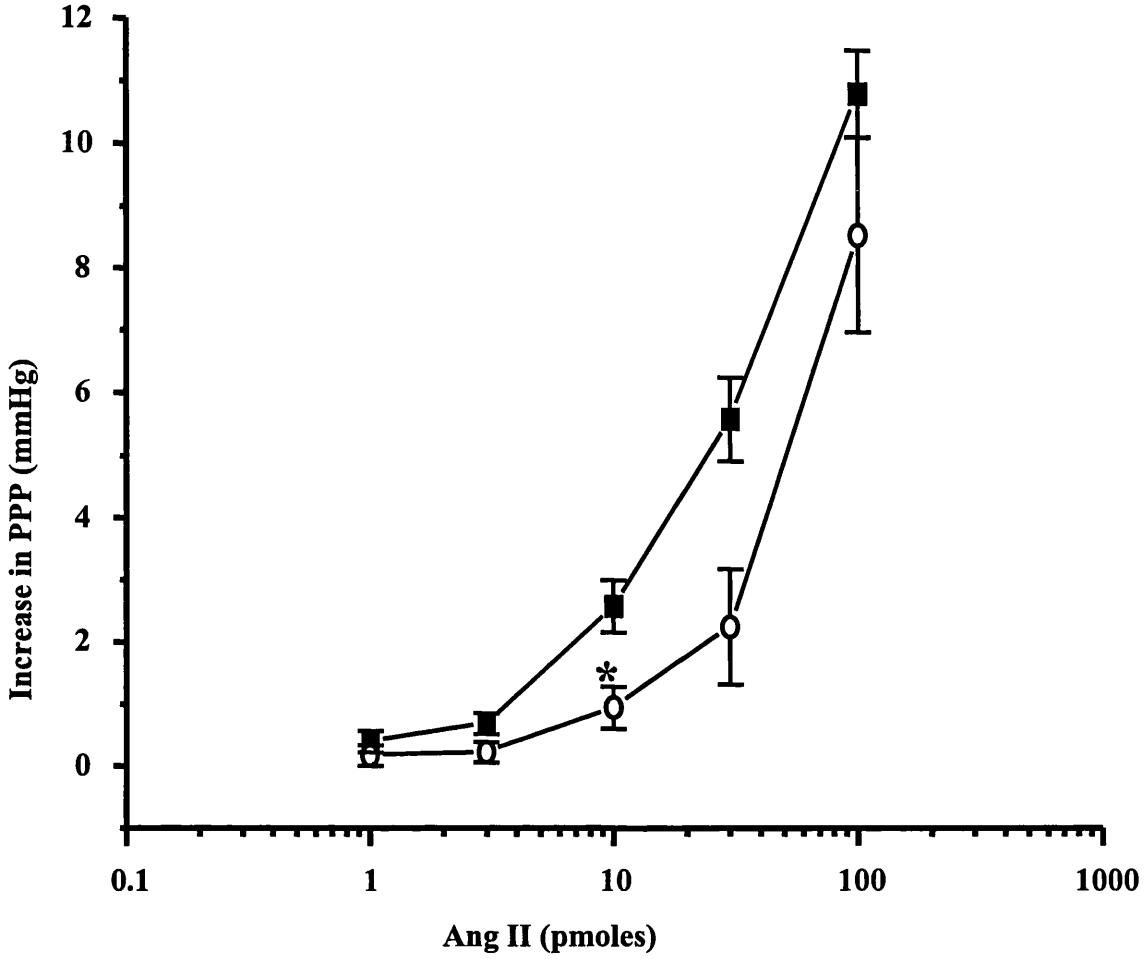

Figure 3.40: Effect of a selective ET_B receptor antagonist, BQ788 on the vasoconstrictor responses to Ang II with ET-1 in normoxic isolated perfused lungs.

Data are presented as increases of PPP (mmHg) to bolus injections of Ang II (1 - 100 pmoles) into the lungs with ET-1 alone (1nM) (■) or with ET-1 plus BQ788 (5 μM) (O). Each point represents mean ± s. e. mean. *P < 0.05 vs. ET-1 controls; unpaired Student's t-test. n = 8 for ET-1 controls and n = 5 for BQ788 treated group.

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3.3.3 Effect of staurosporine and RO-32-0432 on ET-1 sensitization to responses in isolated lungs

Staurosporine, a non-selective PKC inhibitor and RO-32-0432, a selective PKC inhibitor were investigated on ET-1 sensitization of the vasoconstrictor responses to Ang II. As the controls, the vasoconstrictor responses to Ang II were recorded when 1nM ET-1 had been infused for 30 min. After infusion of 1nM ET-1 for 30 min plus infusion of staurosporine or RO-32-0432 for the last 15 min, dose-responses to Ang II were recorded as the treated groups.

Staurosporine reduced the potentiated vasoconstrictor responses to Ang II by ET-1 in the isolated perfused lungs from normoxic rats (Figure 3.41). PPP increases to 100 pmoles Ang II with ET-1 were reduced by staurosporine (100 nM) from 10.8 ± 0.7 mmHg (n = 8) in ET-1 controls into 1.5 ± 0.2 mmHg (n = 3) with ET-1 plus staurosporine ($P < 0.001$). However, RO-32-0432 (100 nM) did not attenuate the potentiation effect of ET-1 on the vasoconstrictor responses to Ang II (Figure 3.42).

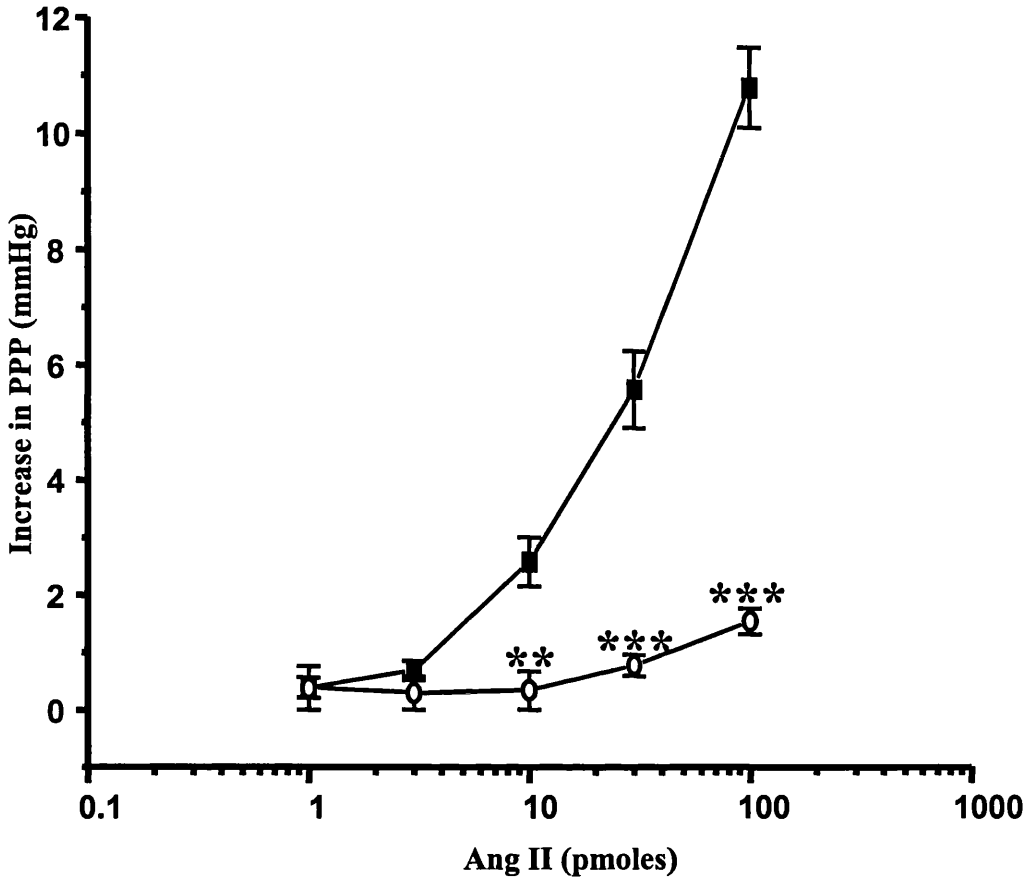

Figure 3.41: Effect of a non-selective PKC inhibitor, staurosporine on the responses to Ang II with ET-1 in normoxic isolated perfused lungs.

Data are presented as increases of PPP (mmHg) to bolus injections of Ang II (1 - 100 pmoles) into the lungs with ET-1 alone (1 nM) (■) or with ET-1 plus staurosporine (100 nM) (○). Each point represents mean ± s. e. mean. **P < 0.01 vs. ET-1 controls, ***P < 0.001 vs. ET-1 controls; unpaired Student's t-test. n = 8 for ET-1 controls and n = 3 staurosporine treated group.

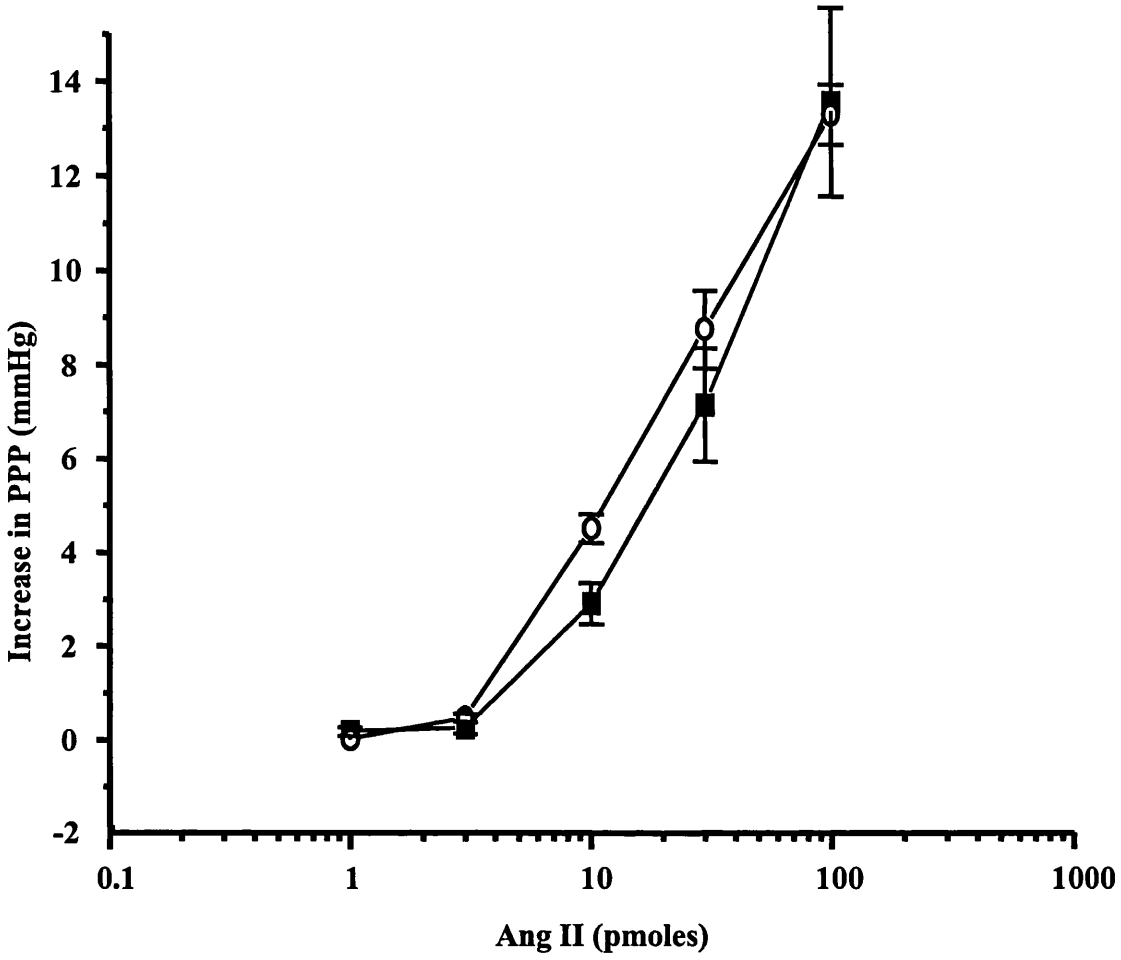

Figure 3.42: Effect of a selective PKC inhibitor, RO-32-0432 on the responses to Ang II with ET-1 in normoxic isolated perfused lungs.

Data are presented as increases of PPP (mmHg) to bolus injections of Ang II (1 - 100 pmoles) into the lungs with ET-1 alone (1nM) (■) or with ET-1 plus RO-32-0432 (100 nM) (○). Each point represents mean ± s. e. mean, n = 4 for each group.

3.3.4 Effects of HOE642 on ET-1 sensitization to vasoconstrictor responses in isolated lungs

HOE642, a selective Na^+/H^+ exchanger inhibitor was tested on ET-1 sensitization of the vasoconstrictor responses Ang II to in isolated perfused lungs. HOE642 (10 μM) had no effect on the potentiation of ET-1 on the vasoconstrictor responses to Ang II (Figure 3.43).

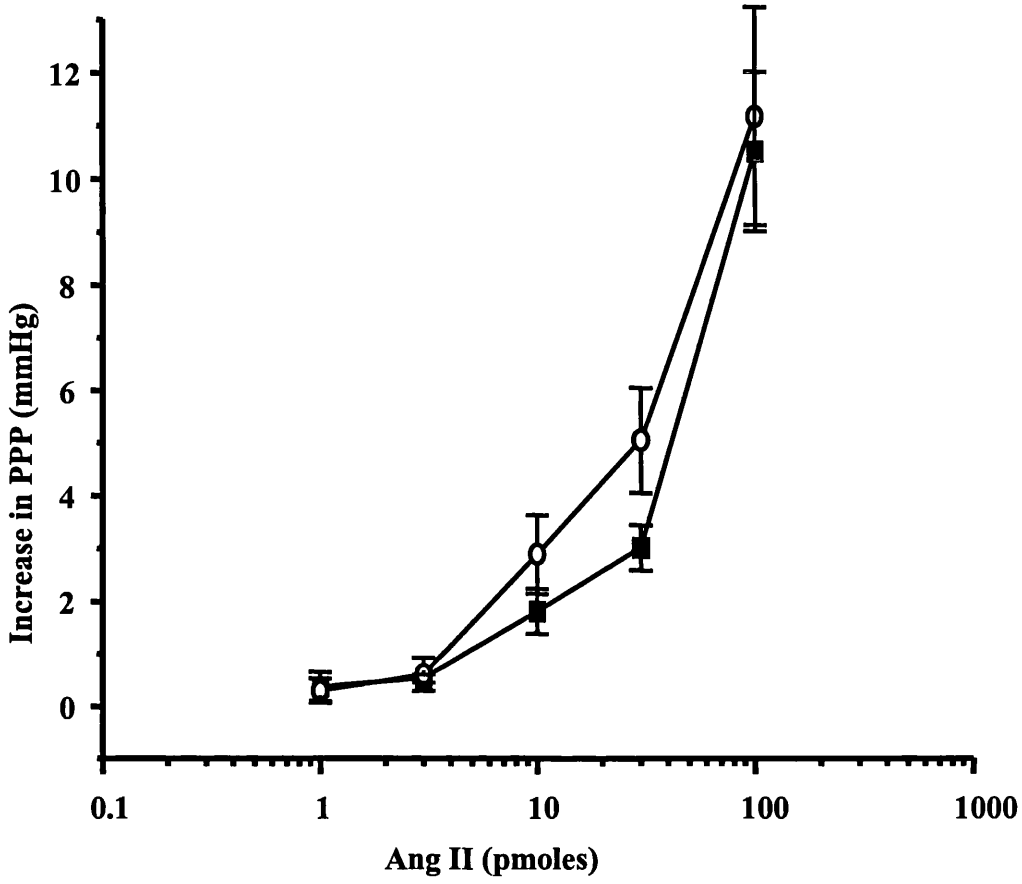

Figure 3.43: Effect of a selective Na^+/H^+ exchanger inhibitor, HOE642 on the vasoconstrictor responses to Ang II with ET-1 in normoxic isolated perfused lungs.

Data are presented as increases of PPP (mmHg) to bolus injections of Ang II (1 - 100 pmoles) into the lungs with ET-1 alone (1nM) (■) or with ET-1 plus HOE642 (10 μM) (O). Each point represents mean ± s. e. mean. n = 4 - 5 for each group.

3.3.5 Effect of ET-1 on responses to PHE in isolated lungs

Vasoconstrictor responses to PHE were also investigated in the presence of ET-1. Similar to Ang II, the dose-response curve of PHE was shifted to the left (Figure 3.44), compared with the control. PPP increases to 1 nmole PHE in the controls and group with ET-1 were 1.9 ± 0.3 mmHg and 2.7 ± 0.2 mmHg, respectively; the responses to 3 nmoles PHE were increased by ET-1 from 2.2 ± 0.4 mmHg in the controls into 3.3 ± 0.4 mmHg ($n = 8$, $P < 0.05$). But ED_{50}s of the two groups were not significantly different, 1.7 ± 0.6 nmoles ($n = 7$) in the controls and 0.6 ± 0.2 nmoles ($n = 4$) in the group with ET-1.

To demonstrate the potentiated vasoconstrictor responses to PHE were due to ET-1, time-matched responses to PHE were also recorded. Bolus injections of PHE were repeated after 30 min, which was the same period as infusion of ET-1. However the second dose-response curve of PHE did not differ from the first one ($P > 0.05$, $n = 5$) (Figure 3.45).

The role of ET receptors in ET-1 sensitization to PHE-induced vasoconstrictor responses in the isolated perfused lungs was also investigated. Neither PD156707 (5 μM) nor BQ788 (5 μM) affected the vasoconstrictor responses to PHE alone. However, both PD156707 and BQ788 shifted the vasoconstrictor response curve to PHE with 1 nM ET-1 to the right (Figure 3.46 & 3.47). PPP increases to 10 nmoles PHE with ET-1 were reduced by PD156707 (5 μM) from 3.86 ± 0.45 mmHg in ET-1 controls to 1.91 ± 0.26 mmHg in PD156707 treated group ($n = 4 - 12$, $P < 0.01$). BQ788 (5 μM) shifted PPP

increases by 10 nmoles PHE with ET-1 from 3.86 ± 0.45 mmHg to 2.01 ± 0.57 mmHg (n = 5 - 12, $P < 0.05$).

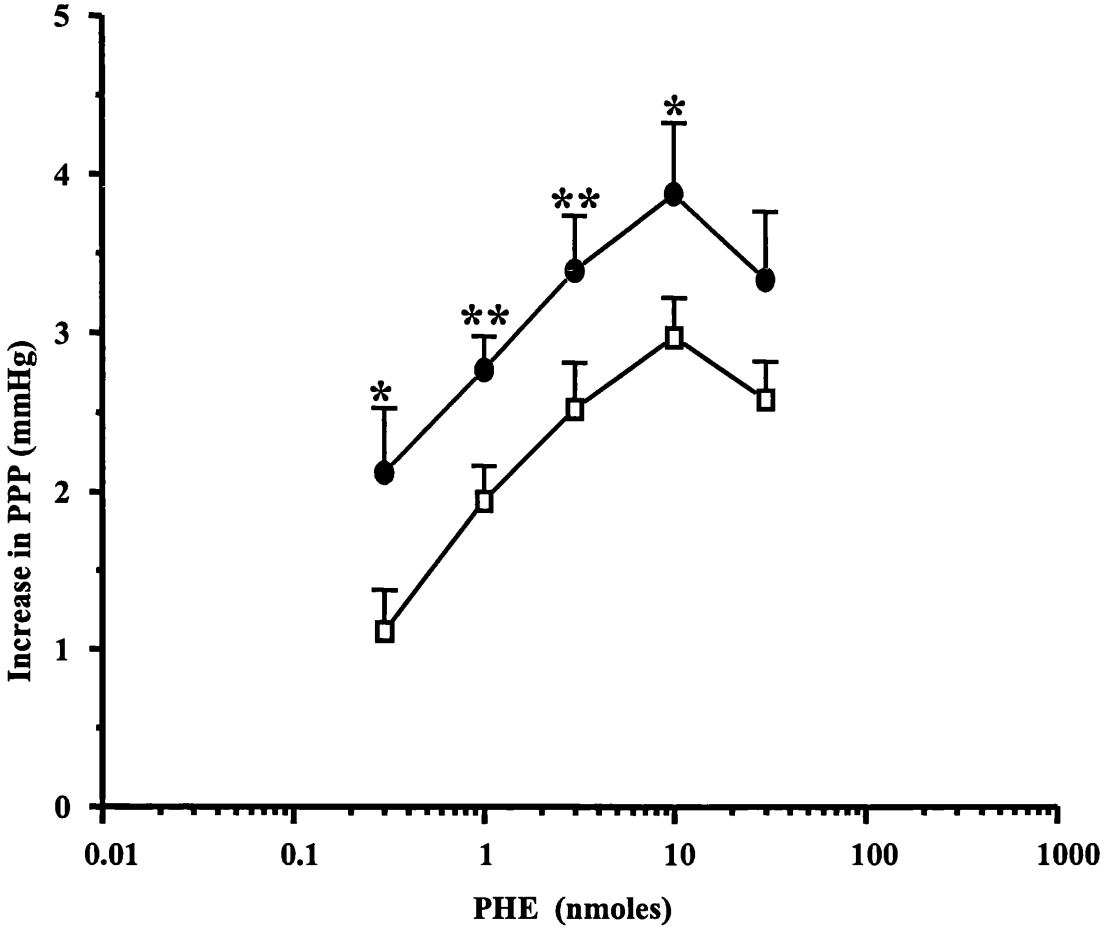

Figure 3.44: Effect of ET-1 on vasoconstrictor responses to PHE in normoxic isolated perfused lungs.

Data are presented as increases of PPP (mmHg) to bolus injections of PHE (0.3 - 30 nmoles) into the lungs in the controls (□) and with ET-1 (1nM) (●) group. Each point represents mean ± s. e. mean. *P < 0.05 vs. the controls; **P < 0.01 vs. the controls; paired Student's t-test. n = 12 for each group.

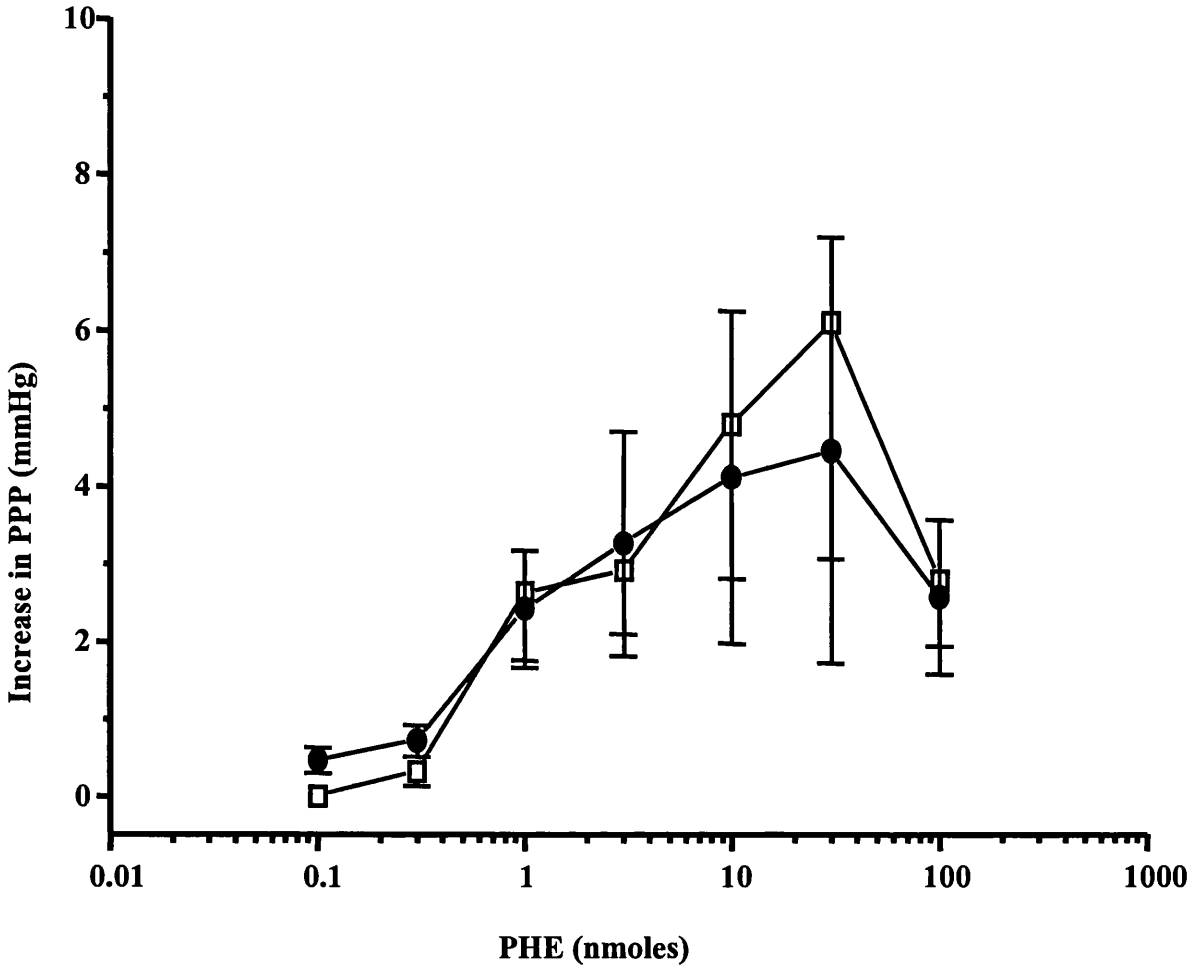

Figure 3.45: Time-matched study of vasoconstrictor responses to PHE in normoxic isolated perfused lungs.

Data are presented as increases of PPP (mmHg) to bolus injections of PHE (0.1 - 100 nmoles) into the lungs in the controls (□) and repeated after 30 min (●). Each point represents mean ± s. e. mean. n = 5 for each group.

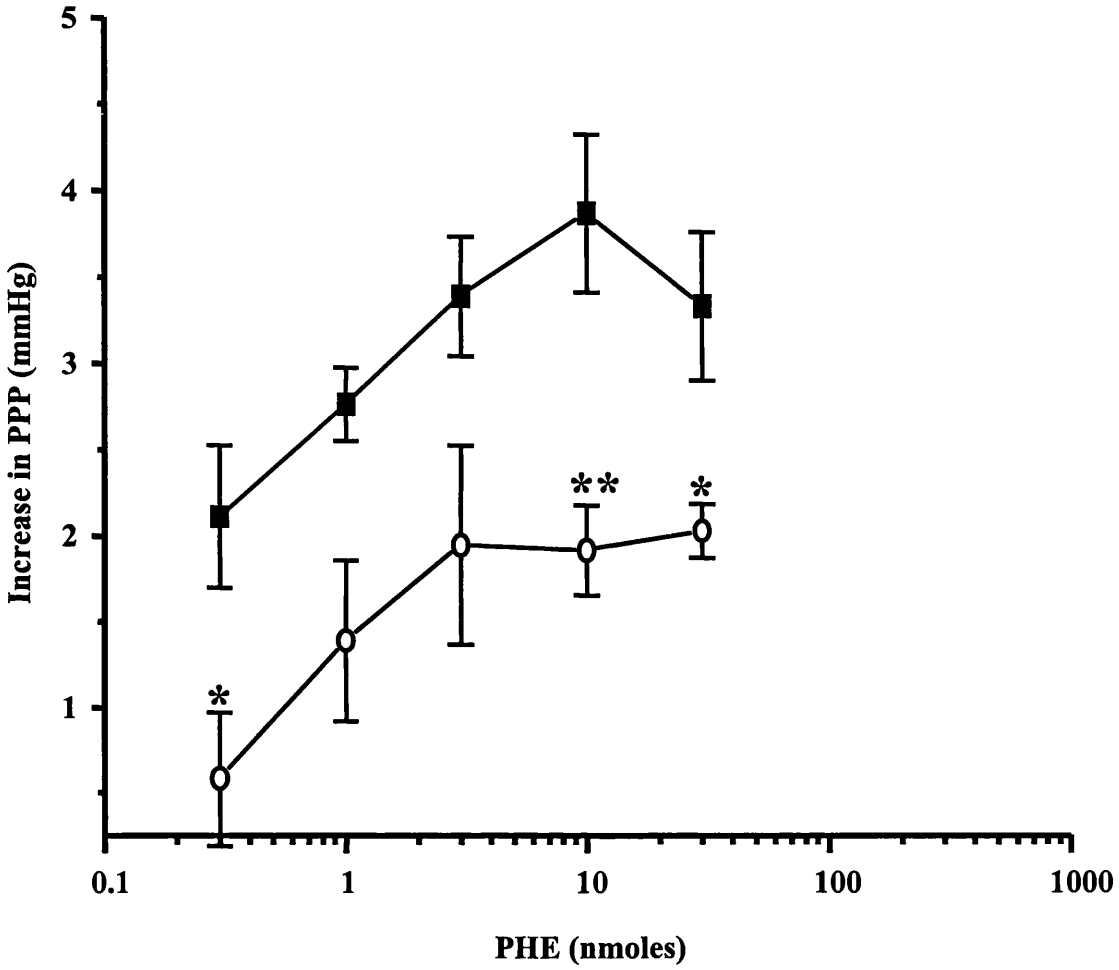

Figure 3.46: Effect of a selective ET_A receptor antagonist, PD156707 on the vasoconstrictor responses to PHE with ET-1 in normoxic isolated perfused lungs.

Data are presented as increases of PPP (mmHg) to bolus injections of PHE (0.3 - 30 nmoles) into the lungs with ET-1 alone (1nM) (■) or with ET-1 plus PD156707 (5 μM) (O). Each point represents mean ± s. e. mean. *P < 0.05 vs. ET-1 controls; **P < 0.01 vs. ET-1 controls; unpaired Student's t-test. n = 12 for ET-1 controls and n = 4 for PD156707 treated group.

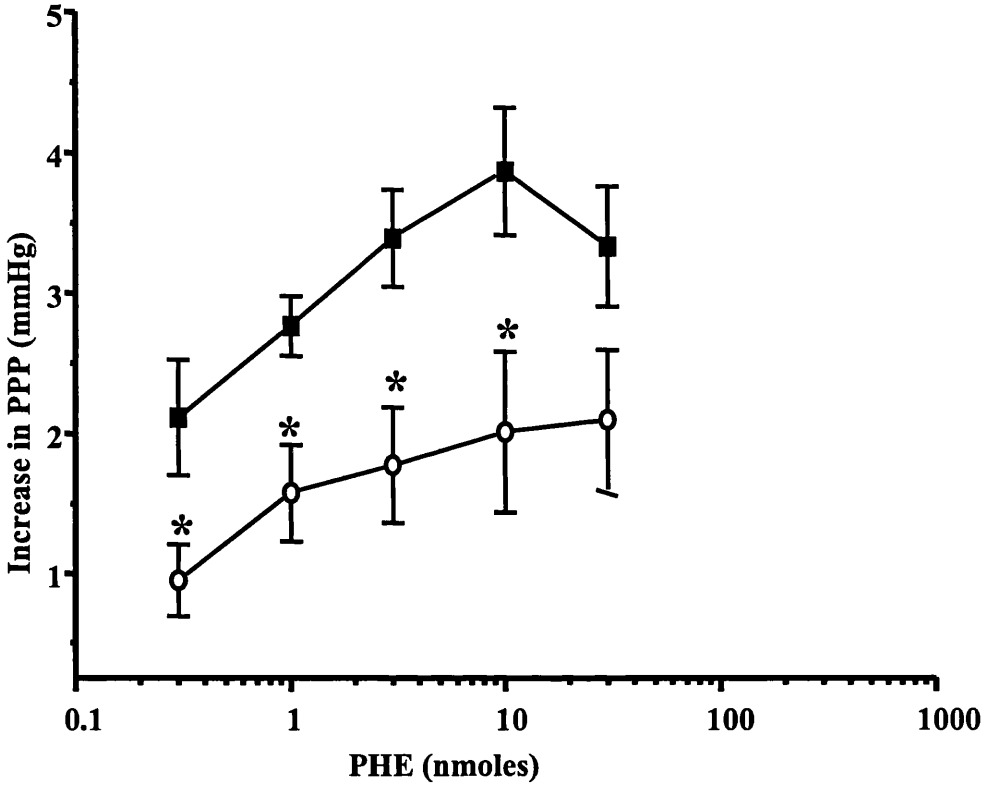

Figure 3.47: Effect of a selective ET_B receptor antagonist, BQ788 on vasoconstrictor responses to PHE with ET-1 in normoxic isolated perfused lungs.

Data are presented as increases of PPP (mmHg) to bolus injections of PHE (0.3 - 30 nmoles) into the lungs with ET-1 alone (1nM) (■) or with ET-1 plus BQ788 (5 μM) (O). Each point represents mean ± s. e. mean. *P < 0.05 vs. ET-1 controls; unpaired Student's t-test. n = 12 for ET-1 controls and n = 5 for BQ788 treated group.

3.3.6 Effect of ET-1 on responses to KCl in isolated lungs

KCl (25 - 400 μmoles) was also tested during infusion of ET-1 in isolated perfused lungs. Dose-responses to KCl were recorded before and after infusing the sub-threshold vasoconstrictor concentration of ET-1 (1nM) for 30 minutes. ET-1 did not change KCl-induced vasoconstrictor responses in the isolated perfused lungs from normoxic rats, $P > 0.05$, $n = 4$ (Figure 3.48).

In order to investigate any effect from nervous system on KCl responses in isolated perfused lungs, prazosin (1 μM), an α_1-adrenoceptor antagonist was infused for 15 min before bolus-injections of KCl. Prazosin had no effect on the vasoconstrictor responses to KCl in isolated perfused lungs. To confirm that 1 μM prazosin is sufficient to block α_1-adrenoceptor on the pulmonary vascular smooth muscle, the vasoconstrictor responses to PHE were recorded in the absence or presence of prazosin in the isolated perfused lungs. prazosin (1 μM) completely abolished the vasoconstrictor responses to PHE in isolated perfused lungs (Figure 3.49 & 3.50).

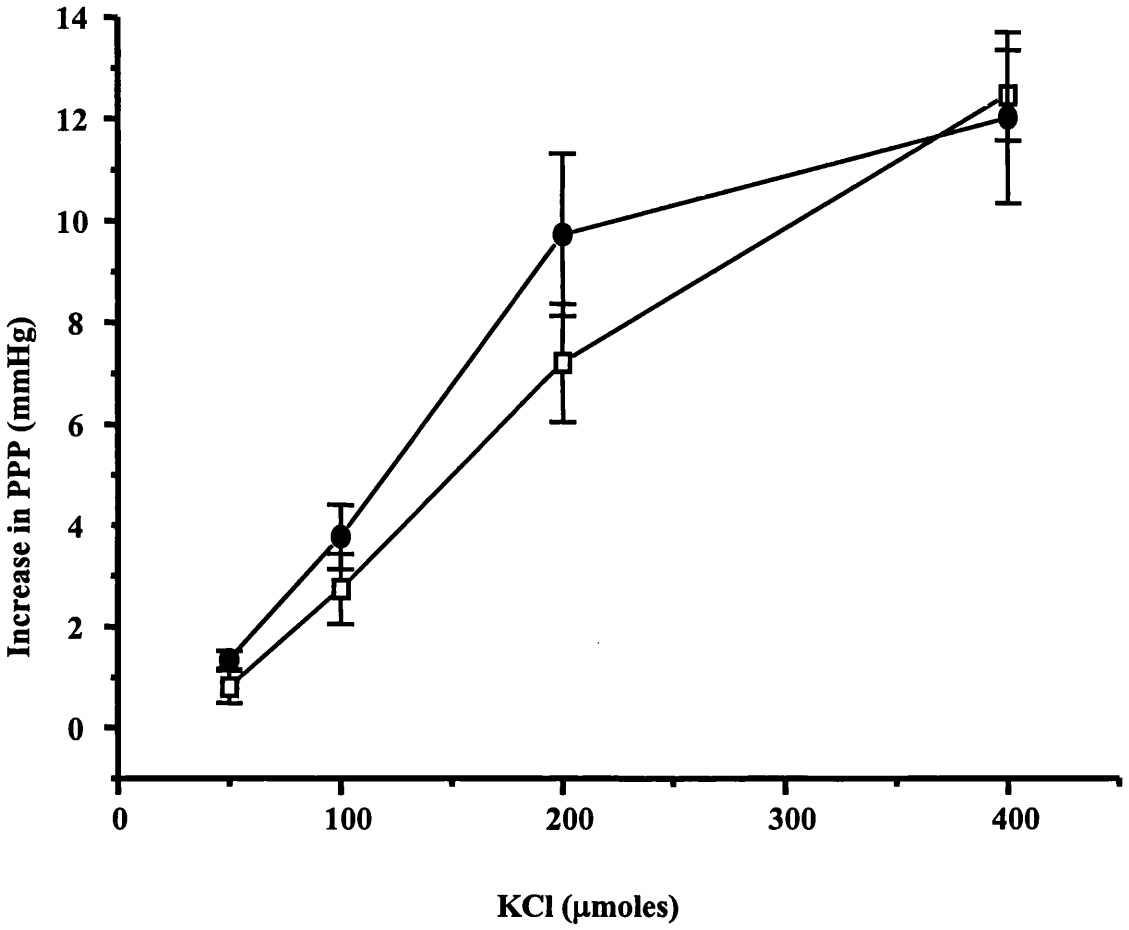

Figure 3.48: Effect of ET-1 on vasoconstrictor responses to KCl in normoxic isolated perfused lungs.

Data are presented as increases of PPP (mmHg) to bolus injections of KCl (25 - 400 μmoles) into the lungs alone (□) or with ET-1 (1nM) (●). Each point represents mean ± s. e. mean. n = 6 for each group.

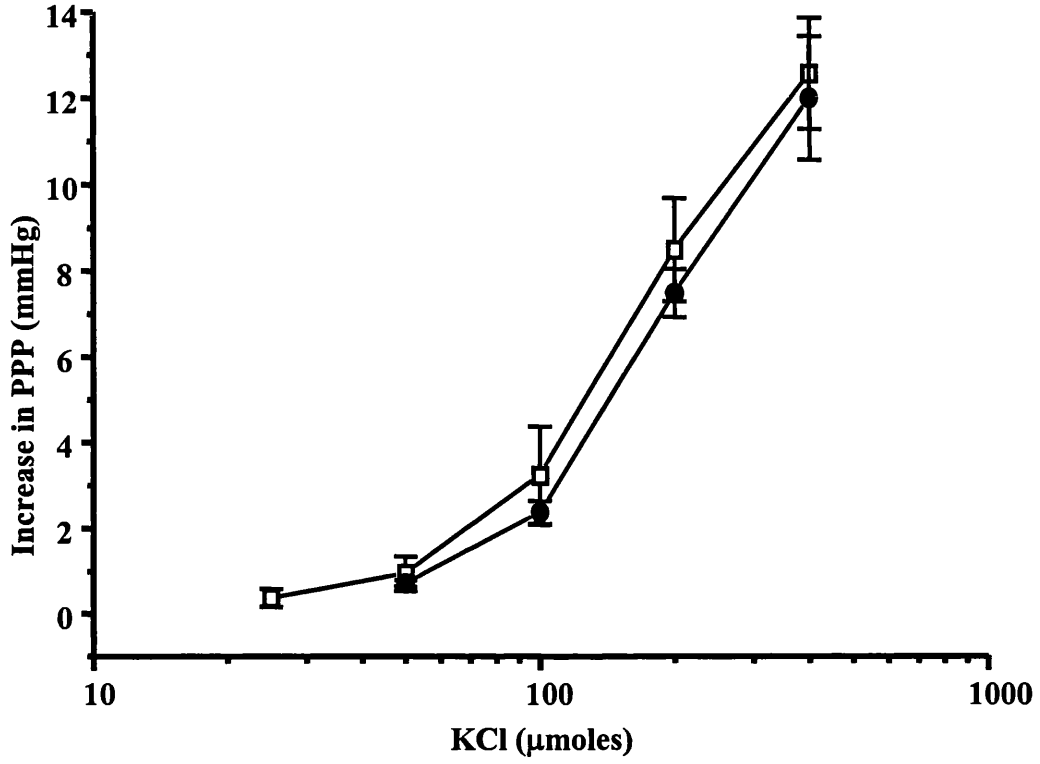

Figure 3.49: Effect of a selective α_1-adrenoceptor antagonist, prazosin on vasoconstrictor responses to KCl in normoxic isolated perfused lungs.

The vasoconstrictor responses to KCl (25 - 400 μmoles) were recorded in the absence (□) or presence (●) of prazosin (1 μM) in normoxic isolated perfused lungs. Each point represents mean ± s. e. mean. n = 5 - 6 for each group.

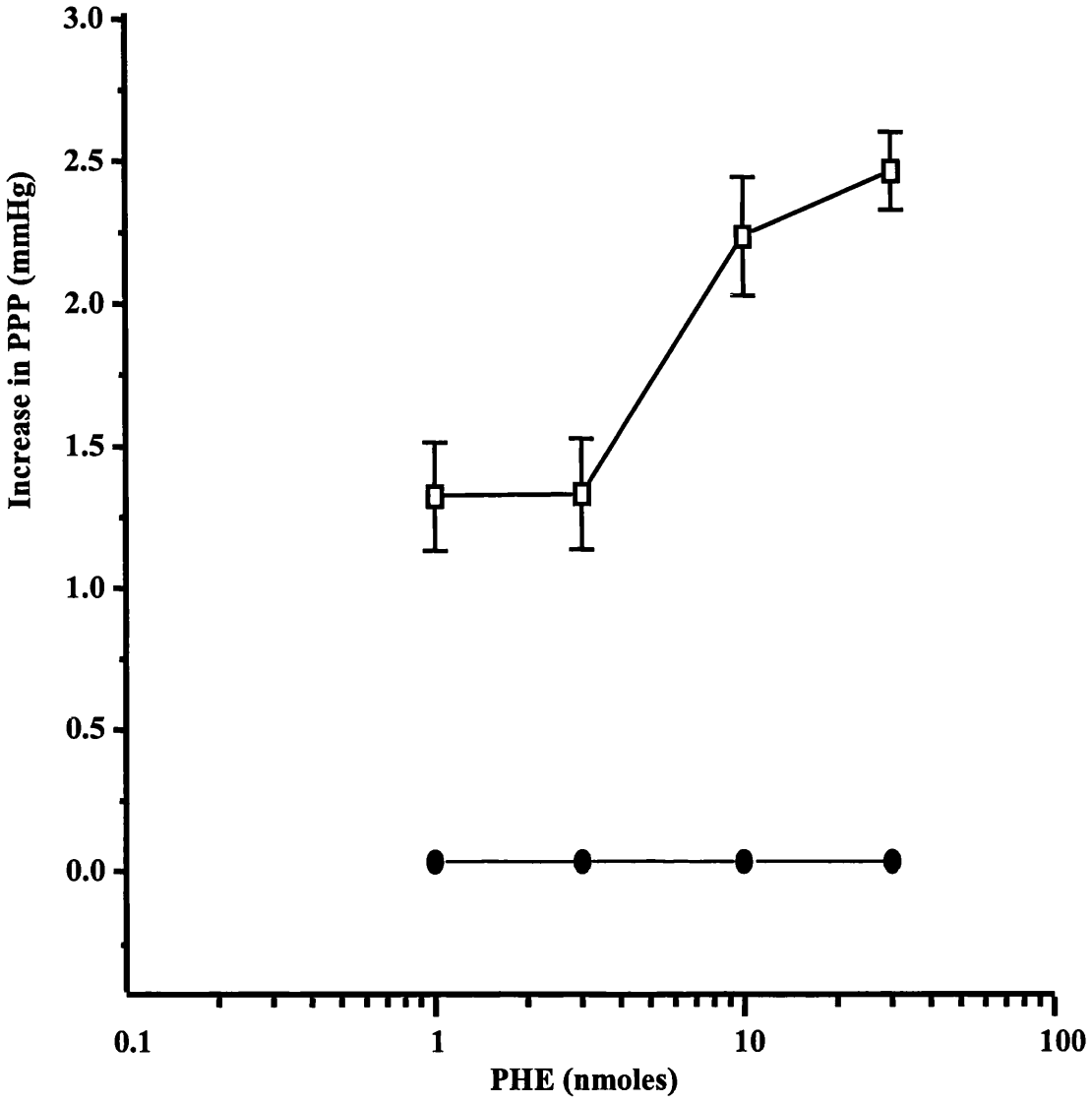

Figure 3.50: Effect of a selective α_1-adrenoceptor antagonist, prazosin on vasoconstrictor responses to PHE in normoxic isolated perfused lungs.

The vasoconstrictor responses to PHE (1 - 30 nmoles) were recorded in the absence (□) or presence (●) of prazosin (1 μM) in normoxic isolated perfused lungs. Each point represents mean ± s. e. mean. n = 4 for each group.

3.3.7 ET-1 sensitization in mesenteric vasculature

For comparison with the results of ET-1 sensitization on vasoconstrictor responses in the lungs, responses in the mesenteric bed were also investigated.

Initial experiments were carried out to determine the sub-threshold pressor concentration of ET-1 in the isolated perfused mesenteric bed. 0.1 nM or 0.3 nM of ET-1 did not change the perfusion pressure significantly, but perfusion pressure significantly increased with infusion of 1 nM ET-1; 21.9 ± 1.0 mmHg (n = 4) in the controls vs. 47.9 ± 6.8 mmHg (n = 4) in the group of 1nM ET-1 infusion, $P < 0.05$ (Figure 3.51).

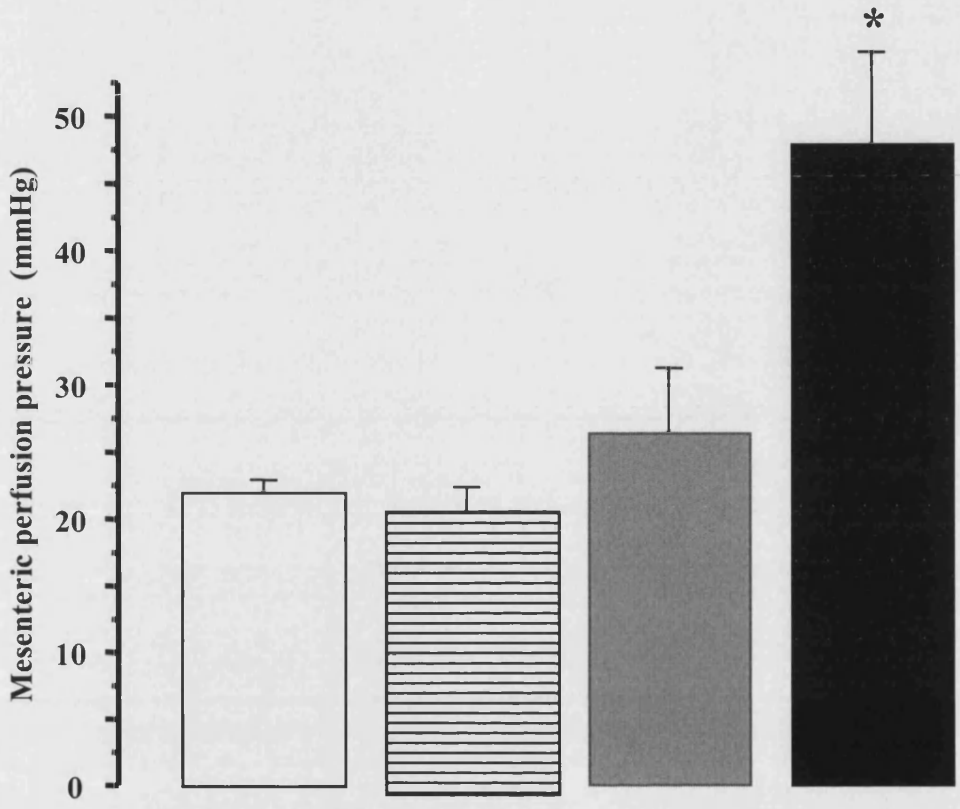

Figure 3.51: Effect of ET-1 on perfusion pressure in isolated mesenteric bed.

The isolated mesenteric bed was perfused without ET-1 (☐) or with 0.1 nM ET-1 (▤), 0.3 nM ET-1 (▒) or 1 nM ET-1 (■) for 15 min. Each column represents mean ± s. e. mean. *P < 0.05 vs. the controls; one way ANOVA followed by Dunnett's test. n = 4 for each group.

Ang II (1pmole – 10nmoles) produced very poor responses in the isolated perfused mesenteric bed. So, only PHE and KCl were chosen as the vasoconstrictors for study of ET-1 sensitization.

The vasoconstrictor responses to 10 nmoles PHE in the isolated perfused mesenteric bed were potentiated when infusing the sub-threshold concentration of ET-1 (0.3nM) (66.6 ± 12.6 mmHg, n = 4), compared with the controls (56.9 ± 12.6 mmHg, n = 4). 10 nmoles PHE-induced vasoconstrictor responses were significantly potentiated by infusing the threshold concentration of ET-1 (1nM) (131.5 ± 12.0 mmHg, n = 4, $P < 0.05$) (Figure 3.52).

Similar results were obtained with KCl (Figure 3.53). The sub-threshold (0.3 nM) or threshold (1 nM) vasoconstriction concentration of ET-1 significantly potentiated the vasoconstrictor responses to KCl (50 μmoles) in the perfused mesenteric beds from normoxic rats. The increases of mesenteric perfusion pressure induced by 50 μmoles KCl were 23.6 ± 1.9 mmHg (n = 3) in the controls, 40.0 ± 1.3 mmHg with 0.3 nM ET-1 ($P < 0.05$, n = 3) and 48.6 ± 2.0 mmHg with 1 nM ET-1 ($P < 0.05$, n = 3).

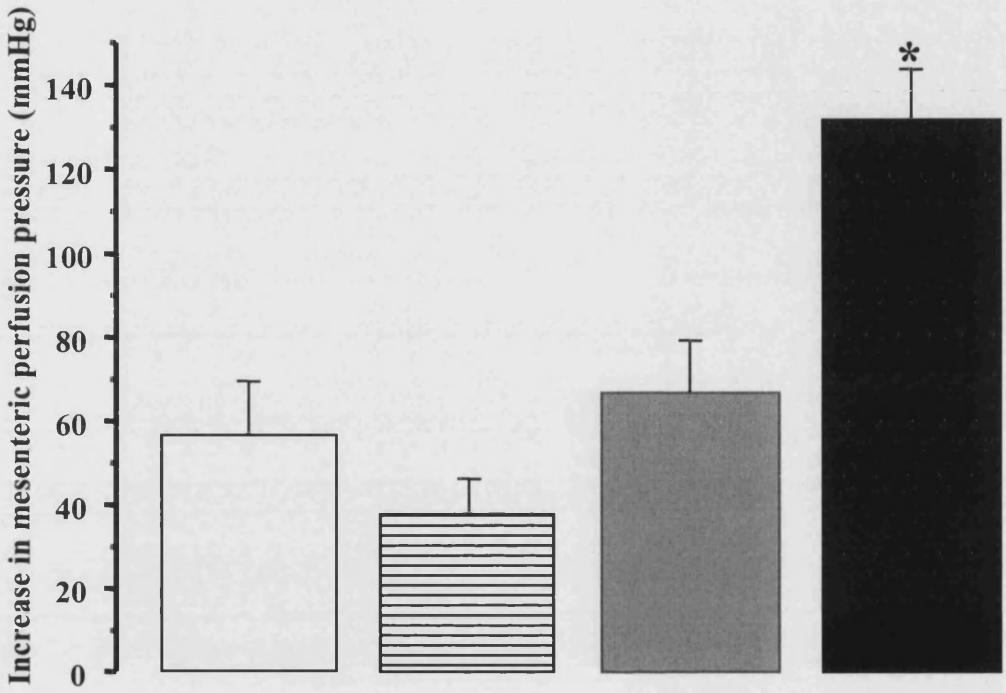

Figure 3.52: Effect of ET-1 on vasoconstrictor responses to PHE in normoxic isolated perfused mesenteric bed.

Data are presented as increases of mesenteric perfusion pressure (mmHg) to bolus injections of PHE (10 nmoles) into the mesenteric artery without ET-1 (☐) or with 0.1 nM ET-1 (▤), 0.3 nM ET-1 (▒) or 1 nM ET-1 (■). Each column represents mean ± s. e. mean. *P < 0.05 vs. the controls; one way ANOVA followed by Dunnett's test. n = 4 for each group.

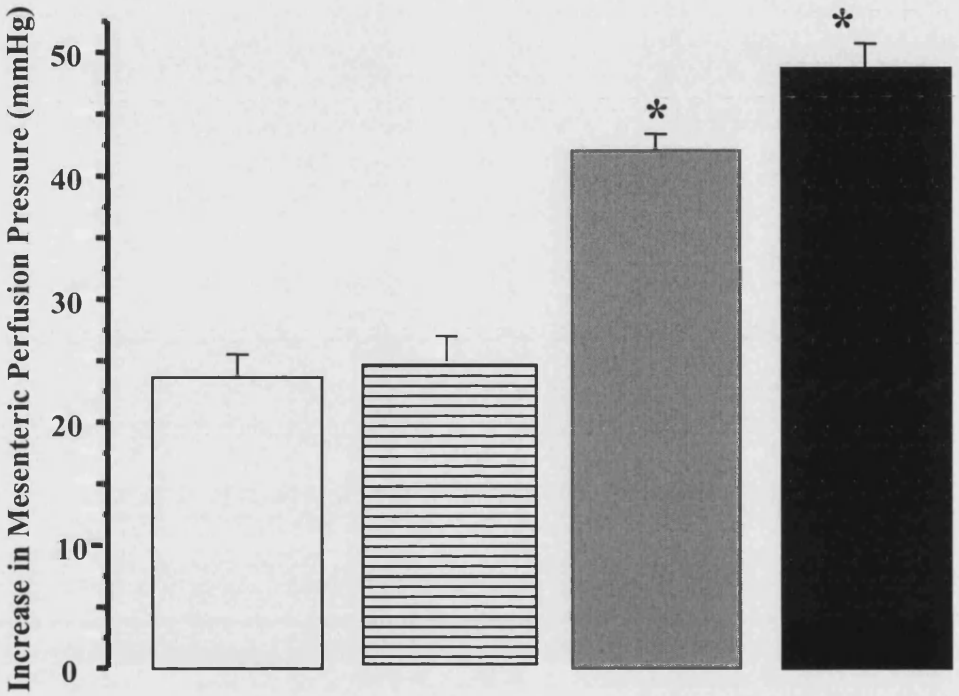

Figure 3.53: Effect of ET-1 on vasoconstrictor responses to KCl in normoxic isolated perfused mesenteric bed.

Data are presented as increases of mesenteric perfusion pressure (mmHg) to bolus injections of KCl (50 μmoles) into the mesenteric artery without ET-1 (☐) or with 0.1 nM ET-1 (▤), 0.3 nM ET-1 (▒) or 1 nM ET-1 (■). Each column represents mean ± s. e. mean. *P < 0.05 vs. the controls; one way ANOVA followed by Dunnett's test. n = 3 for each group.

3.3.8 Studies in permeabilized pulmonary and mesenteric artery rings

For comparison of the contractile responses between pulmonary and mesenteric arteries, pulmonary arteries and mesenteric arteries were set up simultaneously.

Figure 3.54 shows that Ca^{2+} induced contractile responses in permeabilized pulmonary arteries. 0.01 - 10 μM Ca^{2+} (the concentrations of Ca^{2+} that had no effect in non-permeabilized pulmonary arteries) induced concentration-dependent contractions in the permeabilized pulmonary artery rings. This indicated that the vascular smooth muscle in the permeabilized pulmonary arteries had been successfully permeabilized. A similar result was obtained in permeabilized mesenteric arteries with Ca^{2+} buffer.

Caffeine (10 mM) induced transient contractile effects in permeabilized mesenteric arteries (Figure 3.55). The contractile responses induced by caffeine could be reproduced after refilling with 80nM Ca^{2+}/0.2mM EGTA for 10 min after washout. If repeated caffeine (10 mM) was applied again without changing the buffer, the response to caffeine was abolished. Caffeine (10 mM) induced little contractile response in the permeabilized pulmonary arteries, demonstrated in 4 experiment sets.

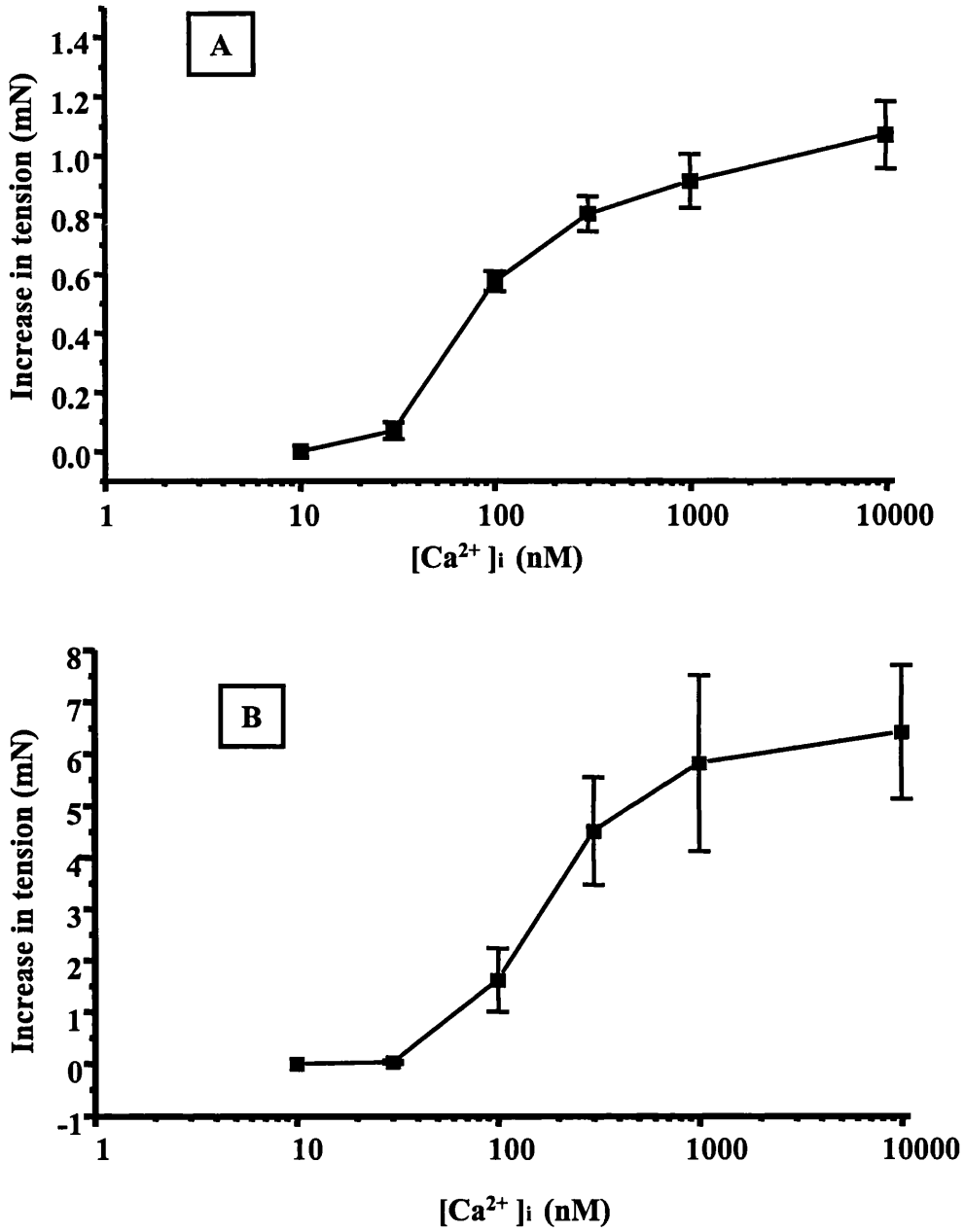

Figure 3.54: $[Ca^{2+}]_i$-tension relationship in α-toxin permeabilized pulmonary and mesenteric arteries.

Data are presented as increases of the vascular tension induced by Ca^{2+} in permeabilized pulmonary (**A**) and mesenteric (**B**) arteries. Ca^{2+} was buffered by 10 mM EGTA and 10 mM Ca^{2+} EGTA. Each point represents mean ± s. e. mean. n = 6 for each point in the pulmonary arteries and n = 7 for each point in the mesenteric arteries.

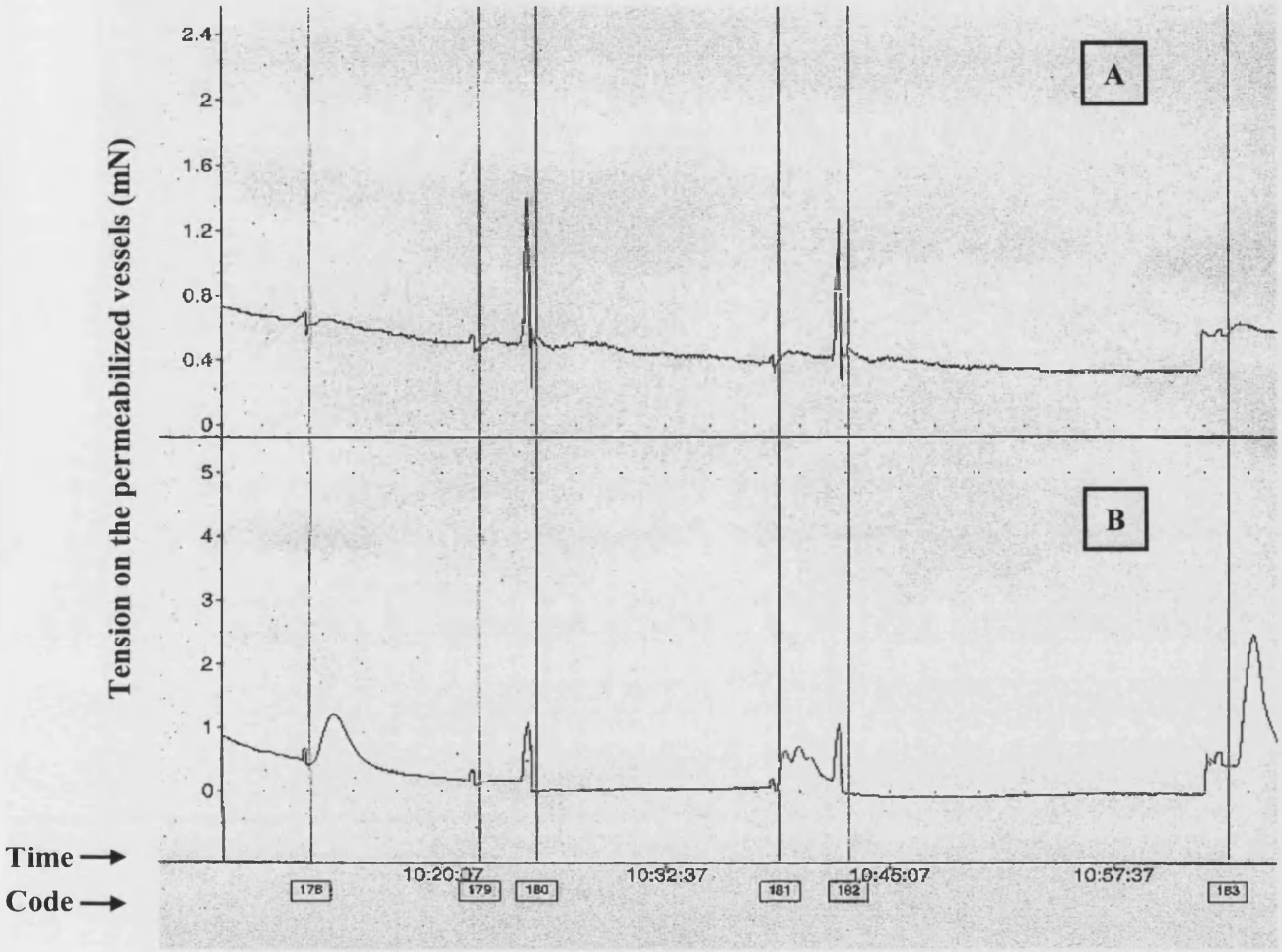

Figure 3.55: Representative traces illustrating the contractile effect of caffeine in α-toxin permeabilized pulmonary and mesenteric arteries.

Bolus-injections of caffeine (10 mM) (code 178, 179, 181 and 183) were added to the permeabilized pulmonary (**A**) and mesenteric (**B**) arteries. The tissues were washed out with Refilling solution (80nM Ca^{2+}/0.2mM EGTA) (code 180 and 182). The responses on code 180 and 182 are artefacts from washout.

3.4 Effect of CH on catecholamine metabolism in isolated lungs

3.4.1 Catecholamine removal in CH isolated lungs

Removal of NA and Adr was estimated by bio-assay of the fraction surviving passage through the lung. NA removal was 49.7 ± 9.9 % (n = 6) in normoxic lungs vs. 47.9 ± 8.2% (n = 6) in CH lungs, $P > 0.05$. Adr removal was significantly lower than NA removal in normoxic lungs, 29.2 ± 5.8%, n = 8 ($P < 0.05$). However CH increased Adr removal to 46 ± 6.6%, (n = 8), significantly higher than Adr removal in normoxic lungs, $P < 0.05$ (Figure 3.56).

For comparison, 5-HT removal was dertermined. 5-HT was almost completely removed from normoxic isolated perfused lungs (82.5 ± 2.8 %, n = 6). CH had no significant effect on this removal (69.8 ± 7.0 %, n = 6), $P > 0.05$ (Figure 3.57).

Further experiments were carried out to determine the mechanism of Adr removal in CH lungs, using $Uptake_1$ and $Uptake_2$ inhibitors. The inhibitors were infused through the isolated perfused lungs for 15 min before infusion of Adr. The removal of Adr in CH lungs did not change in the presence of corticosterone (10 μM), an $Uptake_2$ inhibitor (46 ± 11.3%, n = 4, $P > 0.05$), when compared with CH controls. However cocaine (1 μM), an $Uptake_1$ inhibitor reduced the removal of Adr in CH lungs (6.6 ± 4.8%, n = 5), compared to CH controls, $P < 0.001$ (Figure 3.58).

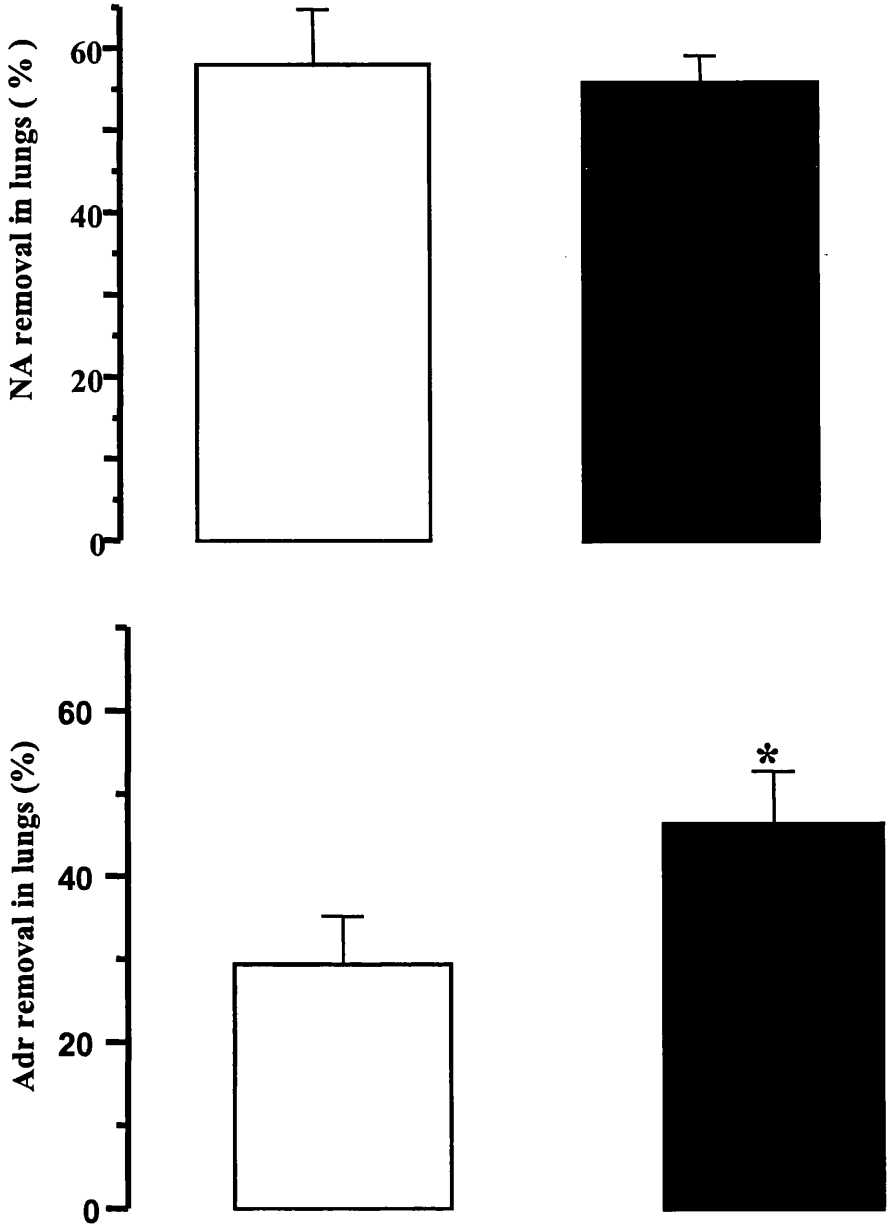

Figure 3.56: NA and Adr removal in normoxic and CH isolated perfused lungs.

NA and Adr removals were measured in the normoxic (□) or CH (■) rat lungs. Each column represents mean ± s. e. mean. * $P < 0.05$ vs. the controls; unpaired Student's t-test. n = 6 for each group in NA removals and n = 8 for each group in Adr removals.

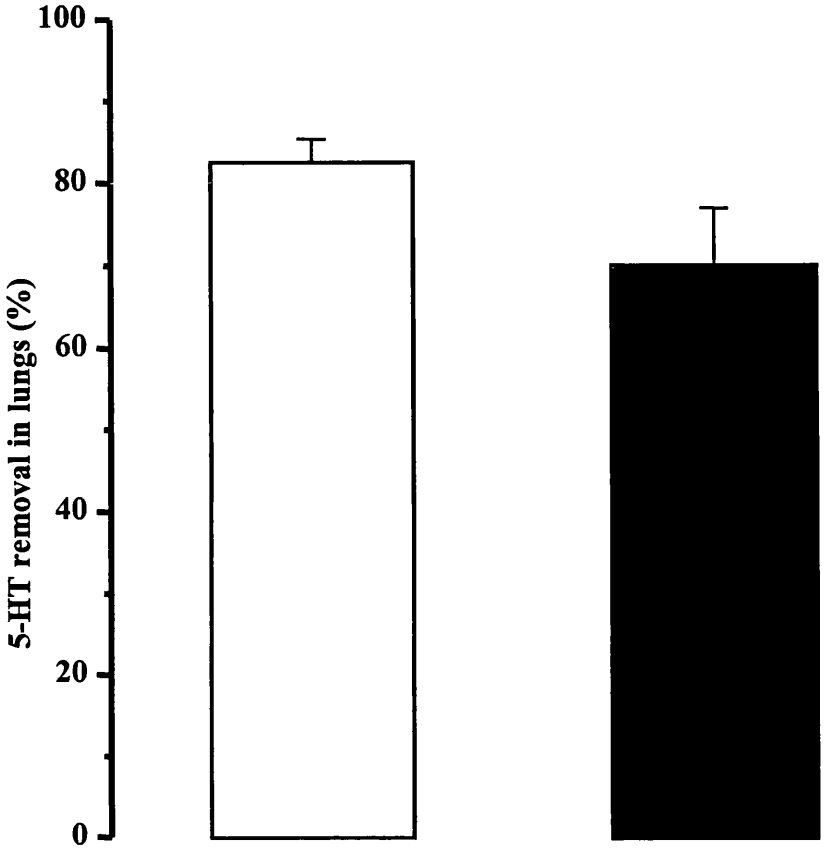

Figure 3.57: 5-HT removal from normoxic and CH isolated perfused lungs.

5-HT removals were measured in the normoxic (□) or CH (■) rat lungs. Each column represents mean ± s. e. mean. n = 6 for each group.

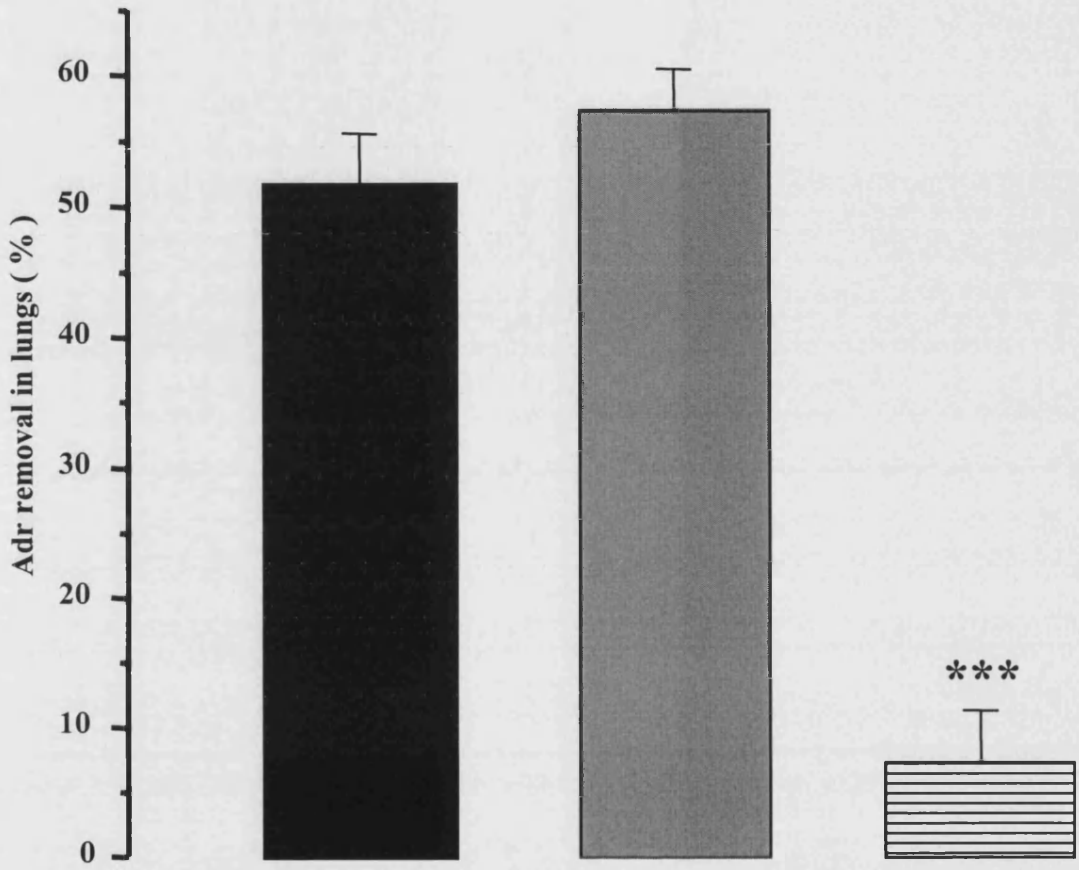

Figure 3.58: Effect of Uptake inhibitors on Adr removal in CH isolated perfused lungs.

Adr removal was measured in CH lungs in the absence of any inhibitor ( ) or presence of corticosterone (10 μM), Uptake2 inhibitor ( ) or cocaine (1 μM), Uptake1 inhibitor ( ). Each column represents mean ± s. e. mean. *** $P < 0.001$ vs. CH controls; unpaired Student's t-test. n = 8 for CH controls, n = 4 for corticosterone treated group and n = 5 for cocaine treated group.

3.4.2 Effects of pulmonary oedema and low oxygen perfusion on Adr removal in isolated lungs

Normally, the isolated perfused lungs were stable over 3 hours. After 3 hours, lung weight increases as pulmonary oedema appears. Adr removal was also checked after lung weight had increased over 30%; lung weights were 2.5 ± 0.3g in the controls vs. 3.9 ± 0.5g in the oedematous lungs. The pulmonary oedema produced in normoxic lungs did not change Adr removal (26 ± 7.3%) when compared to the controls (29 ± 8.1% n = 5, P > 0.05) (Figure 3.59).

When the perfusate solution was aerated by 10% O_2 mixture, Adr removal in CH lungs (38 ± 6.8%, n = 7) was still similar to Adr removal in CH controls (46 ± 6.6%, n = 8, P > 0.05) (Figure 3.60).

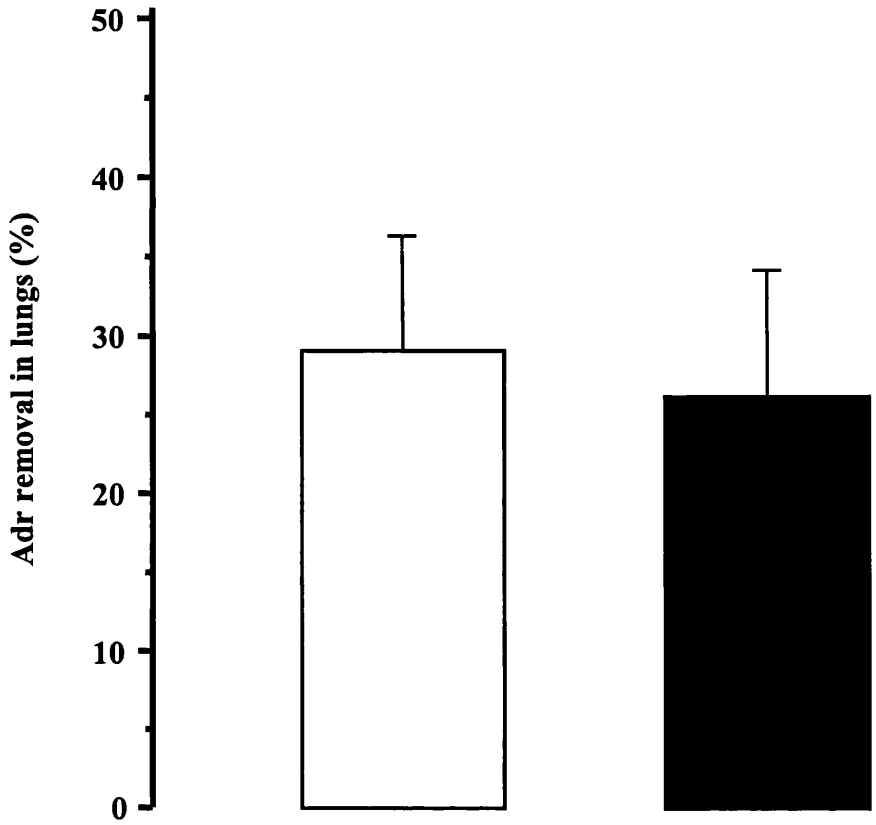

Figure 3.59: Effect of pulmonary oedema on Adr removal in normoxic isolated perfused lungs.

Adr removal was measured in the normoxic lungs (☐) or the lungs with oedema (■). Each column represents mean ± s. e. mean, n = 5 for each group.

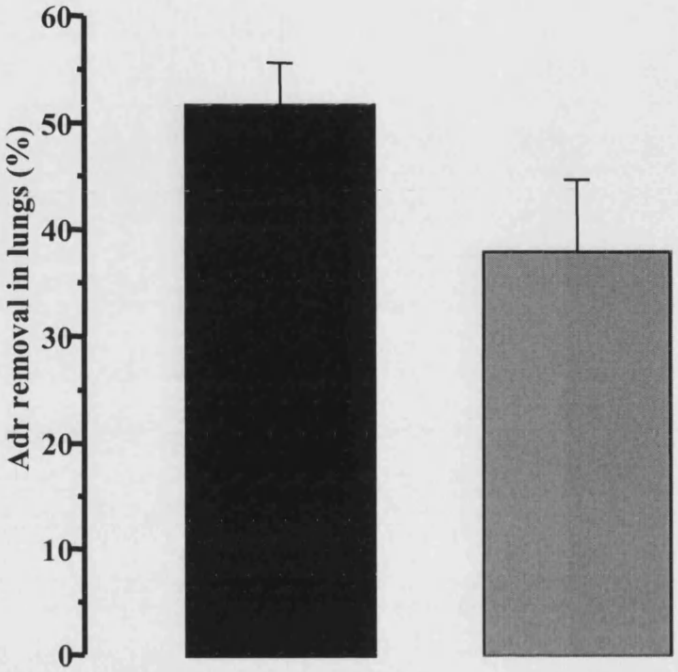

Figure 3.60: Effect of hypoxic perfusion on Adr removal in CH isolated perfused lungs.

Adr removal in CH lungs was not statistically different after switching on 10% O_2 ( ) from 20% O_2 ( ) in the perfusate buffer. The Krebs' solution was bubbled with 10% O_2 or 20% O_2 for 30 min before experimental recording. Each column represents mean ± s. e. mean. n = 7 for 20% O_2 group and n = 8 for 10% O_2 group.

CHAPTER FOUR

DISCUSSION

4.1 Pulmonary vascular hyper-reactivity in isolated lungs of CH rats

CH-induced pulmonary hypertension occurs in residents at high altitude and in chronic lung diseases (Peacock & Raeside, 1998; Rostrup, 1998). Pulmonary hypertension was found 50 years ago, but still attracts many scientists to study it, because the exact mechanism is unknown. In our animal model, rats exposed to hypoxia for 3 weeks showed higher haematocrits and higher pulmonary pressures than in the controls *in vitro*. The ratio of right ventricular weight to total ventricular weight, which was chosen as an index of right ventricular hypertrophy, was also higher in CH rats compared to the normoxic rats. These data confirm the presence of pulmonary hypertension in the CH rats and associated cardiovascular changes similar to those seen in humans.

Exposure to CH potentiated the vasoconstrictor responses to all agonists tested (PHE, Ang II, KCl, U46619, and NA) in the isolated perfused lungs, even though these agonists act through different receptors on the vascular smooth muscle cells. KCl, which acts via membrane depolarization induced Ca^{2+} influx into vascular smooth muscle cells (Ko *et al.*, 1997), also produced potentiated vasoconstrictor responses in CH isolated lungs. The vasoconstrictor responses to PHE or NA were potentiated in CH lungs starting from the threshold doses. The threshold dose-responses to other agonists (Ang II, KCl and U46619) were the same in CH and the control groups; the responses to those agonists were potentiated at higher doses in CH lungs. However, ED_{50}s of all agonists in CH rats were similar to the controls. It is thus unlikely that the changes of responses in CH are caused solely by an increase in receptor number for two reasons: 1) KCl is a non-receptor mediated agonist; 2) the increases to Ang II were not parallel shifted to the left.

Previous work has shown that normoxic perfusion (20%O_2) of CH lungs increased the basal PPP via increased ET synthesis, but hypoxic perfusion (0% O_2) did not change basal PPP (Lal *et al.*, 1999b). In order to investigate whether normoxic perfusion affected pulmonary vasoconstrictor responses in CH lungs, different oxygen concentrations were applied in the perfusate into isolated CH lungs. There was no difference of pulmonary vasoconstrictor responses to PHE between normoxic perfusion (20%O_2) and hypoxic perfusion (10%O_2). This suggests that oxygenation of the perfusate is not the reason for the potentiation of vasoconstrictor responses in CH lungs.

In contrast to pulmonary vasculature, there are no potentiated contractile responses to PHE in aortic rings from CH rats as shown in this study. In uterine artery, a decrease of contractile responses to NA was observed in the CH animals (Hu *et al.*, 1999). Thus, it appears that vascular hyper-reactivity is specific for pulmonary circulation during CH and that the mechanism involved is at a common point in the contractile process.

Enhanced vasoconstrictor responses to Ang II in CH rat lungs have been also reported by other groups (Emery *et al.*, 1981; Jin *et al.*, 1987). ET-1, another important vasoconstrictor for pulmonary vasculature also induced potentiated vasoconstrictor responses in isolated CH lungs (Lal *et al.*, 2000). Monocrotaline-induced pulmonary hypertension exhibited the enhanced vasoconstrictor responses in the pulmonary circulation (Gillespie *et al.*, 1986).

4.2 ET-1 sensitization on vasoconstrictor responses in normoxic isolated lungs

ET-1 as a potent vasoconstrictor is strongly indicated in hypoxic pulmonary hypertension. Hypoxia up-regulates ET converting enzyme activity and induces production of ET-1 (Lal *et al.*, 2000). Also, the number of ET_A receptors in the media of pulmonary resistance arteries increases (Soma *et al.*, 1999). Furthermore, BQ123, a selective ET_A receptor antagonist, attenuated the development of hypoxic pulmonary hypertension in rats *in vivo*, suggesting the involvement of ETs (Bonvallet *et al.*, 1994).

Hence, the potentiated vasoconstrictor responses to agonists in CH lungs observed in the present study might be due to increased production of endogenous ET-1 during CH. Thus, the effects of ET-1 on vasoconstrictor responses in normoxic lungs were carried out to try to mimic the pulmonary hyper-reactivity seen in isolated perfused lungs of CH rats.

Although the plasma concentration of ET-1 is increased in pulmonary hypertension (Stewart *et al.*, 1991; Yoshibayashi *et al.*, 1991), it is still not sufficient to induce direct constriction of pulmonary arteries. Therefore, the concentration of ET-1 utilised for these sensitization studies was deliberately selected as sub-threshold, as determined by measurement of perfusion pressure.

The preliminary experiments were carried out to define this sub-threshold concentration of ET-1. It was found to be 1nM ET-1 in the isolated perfused lung preparation and 0.3

nM ET-1 in isolated perfused mesenteric bed. The threshold concentration of ET-1 do not significantly increase $[Ca^{2+}]_i$ as demonstrated in porcine coronary artery (Nakayama *et al.*, 1991).

Duration of ET-1infusion was found to be important in sensitization to vasoconstrictors in the perfused lung. The time course of sensitization by ET-1 was measured in this study. Infusion of 1nM ET-1 for 30, 45 or 60 minutes significantly potentiated the vasoconstrictor responses to Ang II, and a 30 minutes infusion was chosen as the optimum.

ET-1 infused for 30 minutes potentiated the vasoconstrictor responses to PHE and Ang II in normoxic isolated perfused lungs. Low concentrations of ET-1 can also potentiate the vasoconstrictor responses to NA but not U46619, a TxA_2 mimetic, in porcine aortae, (Scotland *et al.*, 1999). That is because ET-1 shares a common signalling pathway with other vasoactive hormones, such as Ang II, NA and vasopressin in the vascular smooth muscle contraction (Marsden *et al.*, 1989). Furthermore, other agonists whose receptors are G-protein coupled can mutually amplify their vasoconstrictor responses, e.g. α_1-adrenoceptor agonists and 5-HT_2 receptor agonists (Christ *et al.*, 1990).

In the present study, ET-1 significantly potentiated the vasoconstrictor responses to PHE and Ang II in the isolated perfused lung, even though the changes were not dramatic. The explanation is that changes of $[Ca^{2+}]_i$ plays a key role in vascular smooth muscle contraction rather than Ca^{2+} sensitization. Another reason may be because infusion of

ET-1 into lungs not only stimulates ET_A and ET_{B2} receptors on the vascular smooth muscle cells; but also stimulates ET_{B1} receptors inducing NO and PGI_2 release from vascular endothelial cells. This has been shown using L-NOARG, a NO synthase inhibitor plus indomethacin, a cycloxygenase inhibitor, which completely abolished the vasodilation responses induced by ET-1 in isolated perfused lungs (Lal *et al.*, 1996). Generation of such potent vasodilators could attenuate the potentiation effect of ET-1 on the vasoconstrictor responses to agonists. The effects of these antagonists on ET-1 sensitization were not studied.

High K^+-mediated contraction in vascular smooth muscle cells is thought to be through depolarization of membrane potential and an increase of Ca^{2+} influx through L-type Ca^{2+} channels (Zhang & Xiao, 1998). However, it is still possible that high K^+ might stimulate sympathetic nerve terminals in the lung to release NA which would induce vasoconstriction through α_1-adrenoceptors on pulmonary vascular smooth muscle cells. To investigate this possibility, the effect of the α_1-adrenoceptor antagonist prazosin on the vasoconstrictor responses to high K^+ (25 - 400 μmoles KCl) was tested in normoxic lungs. In the presence of prazosin the vasoconstrictor responses to PHE were completely abolished, indicating effective α_1-adrenoceptor blockade; in contrast, the vasoconstrictor responses to high K^+ did not change. Thus NA release from sympathetic nerve terminals does not contribute to the vasoconstrictor responses induced by high K^+.

It is intriguing that the sub-threshold concentration of ET-1 did not potentiate vasoconstrictor responses to high K^+ in normoxic isolated lungs. The main difference

between membrane depolarization induced by high K^+ and agonist-induced activation appears to be related to the activation of membrane bound enzymes, which are stimulated by the latter but not the former. The consequences of this appear to be twofold: 1) agonist-induced Ca^{2+} influx is more effective in raising $[Ca^{2+}]_i$ than Ca^{2+} influx induced by membrane depolarization only (Karaki *et al.*, 1997); 2) agonists enhance the sensitivity of the myofilaments to Ca^{2+}, whereas this probably does not occur if the smooth muscle cells are activated solely by membrane depolarization (Nishimura *et al.*, 1989). In contrast to the vasoconstrictor responses to high K^+ with ET-1 in normoxic lungs, the vasoconstrictor responses to high K^+ were potentiated in CH isolated lungs described previously in this study. So these different results indicate that the pulmonary vascular hyper-reactivity exhibited in CH lungs may not involve ET-1 sensitization to vasoconstrictor responses in pulmonary vasculature.

In order to compare the sensitizing effect of ET-1 in different vasculature, the vasoconstrictor responses to PHE and high K^+ were investigated in the normoxic isolated perfused mesenteric bed. In this tissue, vasoconstrictor responses to high K^+ were significantly potentiated by sub-threshold (0.3 nM) or threshold (1nM) concentrations of ET-1.

The explanation for the difference of high K^+-induced vasoconstrictor responses with ET-1 between the isolated lung and mesenteric bed can be found from the study of Ca^{2+} mobilization in the permeabilized vessels. Caffeine, an opener of CICR ryanodine-sensitive channels (Nishimura *et al.*, 1989; Lagaud *et al.*, 1999), induced contractile

responses in permeabilized mesenteric artery rings, demonstrating the existence of CICR in the rat mesenteric vascular smooth muscle cells. However, in the permeabilized pulmonary artery rings caffeine induced little or no contractile response. Similar results were obtained from measurement of $[Ca^{2+}]_i$. Caffeine (30mM) did not change $[Ca^{2+}]_i$ in the cultured canine pulmonary vascular smooth muscle cells (Shimoda *et al.*, 2000). Pretreatment with ryanodine, an inhibitor of caffeine-sensitive Ca^{2+} release from SR, had no significant effect on the Ca^{2+} increases induced by KCl, PHE or ET-1 in isolated rat pulmonary smooth muscle cells. However thapsigargin, a Ca^{2+}-ATPase inhibitor which blocks the SR Ca^{2+}-ATPase and thus can deplete all Ca^{2+} stores in SR (Maxwell *et al.*, 1998; Ivery *et al.*, 1999), abolished Ca^{2+} mobilization induced by PHE in the same preparation (Hamada *et al.*, 1997).

Generally speaking, Ca^{2+} release from SR in vascular smooth muscle cells is through ryanodine and IP_3 receptors on SR. However, distributions of ryanodine and IP_3 receptors are tissue-dependent. For example, in cardiac myocytes, CICR plays a central role in cardiac excitation-contraction coupling, but not IP_3-induced Ca^{2+} release. Both ryanodine- and IP_3-receptors are ivolved in Ca^{2+} release from SR in mesenteric and aortic smooth muscles (Ito *et al.*, 1986; Lagaud *et al.*, 1999). In pulmonary vascular smooth muscle cells, Ca^{2+} release from SR is through IP_3 receptors rather than ryanodine receptors.

Taken together, these results suggest that high K^+ does not cause CICR in pulmonary smooth muscle cells of rats and ryanodine receptors may not exist in them. The

differential distribution of ryanodine receptors could be one factor for the differential effect of ET-1 on K^+-induced vasoconstrictor responses in the pulmonary and mesenteric vasculature. Further studies are required to investigate in more detail differentiation in Ca^{2+} mobilization between pulmonary artery and systemic resistance vessels.

To determine which ET receptors were involved in the potentiation effect of ET-1 on vasoconstrictor responses in normoxic lungs, the selective ET_A receptor antagonist, PD 156707 (Maguire & Davenport, 1995; Reynolds *et al.*, 1995) and the selective ET_B receptor antagonist, BQ788 (Ishikawa *et al.*, 1994; Webb & Meek, 1997) were used. Both PD156707 and BQ788 blocked the potentiating effect of ET-1 on the vasoconstrictor responses to PHE and ANG II, respectively, demonstrating that both ET_A and ET_B receptors are involved in ET-1 sensitization.

ET receptor sub-types, which mediate ET-1-induced vasoconstriction, vary between species and vascular beds. For example, ET-1 potentiates the pressor responses to NA in perfused rat mesenteric arteries mainly through ET_B receptors (Kita *et al.*, 1998). ET_A receptors are dominant in human pulmonary artery, whereas ET_B receptors are dominant in rabbit pulmonary artery (Fukuroda *et al.*, 1994). In the pulmonary artery tree, the distribution of ET receptor sub-types is regionally different. In rat large extrapulmonary arteries, only ET_A receptors are involved in vasoconstrictor responses to ET-1; in contrast, in intrapulmonary resistance arteries ET_B receptors mediate vasoconstrictor responses to ET-1 (MacLean *et al.*, 1994b; MacLean *et al.*, 1995; Soma *et al.*, 1999). In rat pulmonary artery rings, Evans *et al.* (1999) found that ET-1 induced an increase in

myofilament sensitivity to Ca^{2+} through ET_A receptors, but not ET_B receptors. This is not surprising as the pulmonary arterial rings selected for their experiments had no ET_B receptors (demonstrated by the fact that SX6C, a selective ET_B agonist, did not induce vasoconstrictor responses). It is not difficult to understand that ET-1 sensitizes pulmonary vascular smooth muscle via actions on both ET_A and ET_B receptors in the lungs, as the entire vascular bed is perfused.

Further investigation was carried out into the intracellular signalling pathway, which may be involved in ET-1 sensitization of vasoconstrictor responses. ET-1 activates multiple signalling systems in vascular smooth muscle, including phospholipase C, phospholipase D, phospholipase A2, tyrosine kinases and PKC, as demonstrated in the mesenteric artery (Ohanian *et al.*, 1997). In the canine pulmonary circulation, ET-1 caused pulmonary vasoconstriction through PKC activation (Barman & Pauly, 1995). In cultured rat aortic smooth muscle cells, ET-1 stimulates PKC through DAG (Griendling *et al.*, 1989). In aortic rings, NA-induced contraction was potentiated by ET-1 in the absence of changes in stimulated Ca^{2+} entry, and the PKC inhibitors, staurosporine and calphostin, prevented the potentiation effect of ET-1 (Henrion & Laher, 1993). PKC is considered to inhibit myosin light chain phosphatase and to make smooth muscle cells more sensitive to Ca^{2+} (Nishimura *et al.*, 1989; Weissman *et al.*, 1999). Thus, PKC might be a candidate for myofilamental sensitisation by ET-1 in the isolated lung.

PKC inhibitors were used to investigate the role of PKC in ET-1 in the present study. Ro-32-0432, a selective PKC inhibitor (Birchall *et al.*, 1994; Huang *et al.*, 2000), did not

alter the potentiated vasoconstrictor responses to Ang II by ET-1. Staurosporine inhibited the vasoconstrictor responses to Ang II with ET-1. In fact, the vasoconstrictor responses to Ang II in the presence of ET-1 plus staurosporine were lower than the control group. However, staurosporine is a non-selective kinase inhibitor and can also affect PKA, PKG and myosin light chain kinase (Ivery *et al.*, 1999). PKC involvement in the sensitizing action of ET-1 concluded by other groups is probably because they used such non-selective inhibitors, like H-7 and staurosporine (Nishimura *et al.*, 1992; Wilkinson & Hallam, 1994; Webb & Meek, 1997). The present studies indicate that ET-1 sensitization of the pulmonary artery in the rat lungs is not related to PKC activation.

pHi is another important factor in vascular smooth muscle cells which can modulate vascular tone (Krampetz & Rhoades, 1991). Increase or decrease of pHi in vascular smooth muscle cells would, respectively, potentiate and blunt vasoconstrictor reactivity by changing Ca^{2+} sensitivity in the cells (Raffestin & McMurtry, 1987). pHi can be changed via extracellular pH or the action of agonists (Smith *et al.*, 1998). The Na^+/H^+ exchanger plays an important role in controlling pHi by exchanging intracellular H^+ for extracellular Na^+. The resultant intracellular alkalinization enhances Ca^{2+} sensitivity to vasoconstrictors. In rabbit cardiomyocytes, contraction induced by ET-1 was inhibited by HOE642, a selective Na^+/H^+ exchanger inhibitor, without a significant effect on $[Ca^{2+}]_i$ (Wang *et al.*, 2000). This proves that ET-1 does increase Ca^{2+} sensitivity via the Na^+/H^+ exchanger. However, HOE642 failed to inhibit the potentiation effect of ET-1 on the vasoconstrictor responses to Ang II in normoxic isolated perfused lungs in this study. A Na^+/H^+ exchanger does exist in pulmonary arterial smooth muscle cells demonstrated in

guinea pig and ferret and cat (Quinn *et al.*, 1991; Farrukh *et al.*, 1996; Madden *et al.*, 2001). Furthermore, it has been demonstrated that Na^+/H^+ exchanger is present in both large and small pulmonary arteries and plays an important role in the regulation of pHi in the pulmonary circulation (Madden *et al.*, 2001). However, ET-1 sensitization in the pulmonary circulation does not appear to involve the Na^+/H^+ exchanger and regulation of pHi.

Tyrosine kinase is also not involved in ET-1 sensitization in rat permeabilized pulmonary arterial rings, as demonstrated by Evans *et al.* (1999).

Vascular smooth muscle contraction depends on myosin light chain phosphorylation in the cells. The activities of myosin light chain kinase and myosin light chain phosphatase decide the extent of myosin light chain phosphorylation. Myosin light chain kinase activity depends on $[Ca^{2+}]_i$. In contrast, inhibition of myosin light chain phosphatase, which decreases the dephosphorylation of phosphorylated myosin light chain, can indirectly increase the phosphorylation of myosin light chain and does not depend on $[Ca^{2+}]$. Rho-kinase phosphorylates myosin light chain phosphatase and therefore inhibits its activity (Weissmann *et al.*, 1999). Robertson *et al.* (2000) have demonstrated that Y-27632, a specific inhibitor of Rho-activated kinase, prevented HPV in isolated intrapulmonary artery rings and isolated perfused lungs from rats (Robertson *et al.*, 2000). This indicates that increased Ca^{2+} sensitivity plays a role in HPV. Therefore, Rho-kinase could be a candidate for ET-1 sensitization of vasoconstrictor responses in the isolated perfused lung.

4.3 Effect of inhibitors of ET-1 synthesis on pulmonary vascular hyper-reactivity in CH isolated lungs

To elucidate whether endogenous ET-1 is responsible for pulmonary vascular hyper-reactivity in CH lungs, ET receptor antagonists and an ET converting enzyme inhibitor were used.

As the α_1-adrenoceptor is an important receptor in the regulation of pulmonary tone (Salvi, 1999), PHE, an α_1-adrenoceptor agonist was chosen first. PD156707 (5 μM), another selective ET_A receptor antagonist did not alter the vasoconstrictor responses to PHE in CH isolated perfused lungs. Experiments had shown that vasoconstrictor responses to ET-1 were effectively blocked in normoxic isolated perfused lungs, indicating that the concentration of PD156707 was sufficient to block ET_A receptors. BQ788, a selective ET_B receptor antagonist, also had no effect on the vasoconstrictor responses to PHE in CH lungs.

Conversion of big ET-1 to mature ET-1 is essential for the expression of full biological activity of ET-1 (Hisaki *et al.*, 1994). Phosphoramidon, an ET converting enzyme inhibitor prevents the conversion of big ET-1 into ET-1 (Hisaki *et al.*, 1994; Held *et al.*, 1997). However, phosphoramidon (10 μM) (Smith *et al.*, 1997) had no effect on the vasoconstrictor responses to PHE.

BQ123 however had no effect on the vasoconstrictor responses to Ang II or KCl in CH

lungs. The vasoconstrictor responses to KCl in CH lungs were also not affected by BQ788.

Accordingly, these studies do not support the hypothesis that endogenous ET-1 production plays a role in pulmonary vascular hyper-reactivity during CH.

4.4 Relationship between pulmonary vascular remodelling and pulmonary vascular hyper-reacitivity induced by CH

Pulmonary vasculature shows pulmonary vasoconstriction during the early stage of hypoxic pulmonary hypertension, and is gradually supplanted by progressive structural changes, so called 'muscularization' of pulmonary arteries (Meyrick & Perkett, 1989). This new muscle encroaches on the vascular lumen and makes it narrow. This increase in vascular smooth muscle has been cited as the reason for the augmentation of vasoconstrictor responses to different agonists in CH lungs (Emery *et al.*, 1981).

An approach was designed to determine whether the increased vascular reactivity in CH lungs is due to 'muscularization'. This involved measuring the time-course of onset and offset of vascular hypertrophy during and after CH and measuring changes in vascular reactivity at these times.

After 21 days hypoxia the vasoconstrictor responses to PHE, Ang II and KCl were all significantly potentiated. The time course of onset of these effects appeared to be similar for each of the agonists. This would support a correlation between pulmonary hyper-reactivity and 'muscularization'. However discrepancies were noted in these two parameters when animals were returned to normoxic conditions (recovery). The vasoconstrictor responses to PHE in the isolated perfused lungs returned to control levels after 21 days recovery. However, the vasoconstrictor responses to Ang II and KCl in the isolated lungs were still augmented after 21 days recovery.

Extensive histological examination of pulmonary arteries was carried out in lungs taken after 21 days hypoxia and 21 days recovery and compared with the age-matched controls. The ratio of pulmonary wall thickness was significantly higher after 21 days hypoxia, compared to normoxic vessels. This hypertrophy was still present after 21 days recovery.

Right ventricular hypertrophy is a well-established index for the degree of pulmonary hypertension, because a close correlation between wall thickness of small arteries and the degree of right ventricular hypertrophy has been noticed (Hislop & Reid, 1976). Ratios of right ventricular to total ventricular weight were significantly increased after 21 days hypoxia and were still significantly higher than control after 21 days recovery. This data suggests that regression of pulmonary vascular remodelling and right ventricular hypertrophy takes place over a much longer period, more than 21 days after animals transferred from 21 days hypoxic condition to normoxia in this study.

It would appear that pulmonary vascular remodelling correlates well with the vasoconstrictor responses to Ang II and KCl. However, PHE-induced vasoconstrictor responses regress more rapidly than vascular hypertrophy. This suggests a different mechanism is involved in the potentiation of PHE responses. The density of α_1-adrenoceptor was up-regulated in pulmonary artery during hypoxia (Xie *et al.*, 1991); and the responses to PHE and NA were shifted to the left in the isolated lungs during CH, described before. Thus, an increase of α-adrenoceptor number could be involved in the potentiation of vasoconstrictor responses to α-adrenoceptor agonists during CH.

Results from a different study also suggest that pulmonary vascular remodelling in CH lungs does not parallel the alteration of pulmonary vascular function. When pulmonary vascular remodelling in CH was attenuated by an angiotensin converting enzyme inhibitor, perindopril, the potentiated vasoconstrictor responses to 5-HT or U46619 still persisted in pulmonary artery rings (Jeffery & Wanstall, 1999). This suggests that the proliferation of vascular smooth muscle alone cannot account for the pulmonary vascular hyper-reactivity in hypoxic pulmonary hypertension.

Apart from muscularization, another possible factor on pulmonary vascular hyper-reactivity during CH is membrane potential of pulmonary artery smooth muscle cells. In adult pulmonary artery smooth muscle cells, K^+ channels determine the membrane potential and both acute and chronic exposures to hypoxia cause K^+ channel inhibition and then membrane depolarisation (Archer *et al.*, 1998). The membrane depolarisation brings the membrane potential closer to the threshold for activation of L-type Ca^{2+} channels (Yuan *et al.,* 1990; Evans *et al.,* 1998). Therefore, vasoconstrictors, e.g. PHE, Ang II and KCl could induce extracellular Ca^{2+} influx more easily during CH.

In order to obtain more detailed knowledge of the mechanisms underlying the potentiated responses in CH lungs, studies were undertaken using rings of pulmonary artery from the different regions of pulmonary artery tree. The physiological and pharmacological characteristics of large and small pulmonary arteries are heterogeneous. Morphological studies also have shown that the structures in the wall of pulmonary conduit arteries are

different from those in the wall of pulmonary muscular arteries (Sasaki *et al.*, 1995). There is great heterogeneity in the phenotype of the vascular smooth muscle cells in pulmonary artery tree. K^+ channels are one example of the discrepant distributions in the pulmonary artery tree (Post *et al.*, 1992; Albarwani *et al.*, 1995). The heterogeneity in distribution of K^+ channels may explain some of the discrepancies in vascular reactivity noted between pulmonary resistance and conduit arteries in response to hypoxia. Pulmonary resistance arteries respond to hypoxia by inhibition of whole cell K^+ current and depolarisation of the membrane leading to Ca^{2+} influx and the vascular smooth muscle constriction (Albarwani *et al.*, 1995). Pulmonary conduit arteries are more akin to systemic arteries and respond to hypoxia by potentiation of K^+ current and hyperpolarisation of the membrane and therefore the vascular smooth muscle relaxation (Albarwani *et al.*, 1995). That is because pulmonary resistance artery smooth muscle cells have a higher density of K_v channels, which exhibit oxygen sensitivity by their K^+ channel inhibition (Post *et al.*, 1992). Conversely, in pulmonary conduit arteries there is a higher proportion of K_{ca} channels, which increase whole cell K^+ current during hypoxic exposure. Conduit and resistance pulmonary myocytes also differ in their voltage gated (L-type) Ca^{2+} channel response to hypoxia. In the conduit arteries hypoxia inhibits Ca^{2+} current whereas in resistance arteries hypoxia increases Ca^{2+} influx (Brij & Peacock, 1998).

For comparison between large and small pulmonary arteries, all vasoconstrictor responses induced in pulmonary arterial rings were standardized by the contractile responses to 50 mM KCl in the same preparation. This kind of standardization allows

comparison of the vasoconstrictor responses to the same agonist in different sizes of vessel. After standardization, concentration-dependent contractions to KCl were exactly the same in large and small pulmonary artery rings. Vasoconstrictor responses to Ang II were also similar between the two groups, even though the responses to Ang II in the small pulmonary artery rings were slightly smaller. In contrast, the vasoconstrictor responses to PHE were much smaller in the small pulmonary arteries than in the large pulmonary arteries.

Previous studies have also demonstrated that the vascular reactivity to vasoconstrictors is not uniform along the pulmonary vasculature (Lal *et al.*, 1999a). α_1-Adrenoreceptors are expressed on most vascular smooth muscle cells, and their sub-types are distributed in a pattern that is specific for functionally distinct vessel types. The population of these receptors and their sub-types vary greatly, not only in different vessels but also at different levels within the same vascular tree, thereby exhibiting regional variations in their reactivity to various agonists (Salvi, 1999). Therefore, different receptor distributions and/or different intrinsic contractility between the large and small pulmonary arteries might be the reason for the regional variations of the vasoconstrictor responses in the pulmonary artery rings shown in this study.

During CH, the vasoconstrictor responses to 5-HT were significantly increased in the pulmonary resistance artery rings, compared to the controls, whereas the responses to KCl and PHE were unchanged and Ang II-induced responses were reduced in the pulmonary resistance arteries from CH rats. For ET-1, there is an increase in the

vasoconstrictor responses to ET-1 in the small muscular pulmonary arteries in CH rats. In the large elastic arteries of CH rats, depending on experimental conditions, both increases and decreases in responsiveness to ET-1 have been reported (Bialecki *et al.*, 1998). Previous work in our laboratory has reported that in pulmonary artery rings, CH enhanced the vasoconstrictor responses to PHE (Lal *et al.,* 1999a). However, there were no enhanced responses to Ang II or ET-1 in main pulmonary rings from CH rats (MacLean *et al.*, 1995; Jeffery & Wanstall, 1999; Lal *et al.*, 1999a). Pulmonary arterial responses to 5-HT are markedly enhanced at all levels of the pulmonary arterial circulation from CH rats (MacLean, 1999a). In extralobar pulmonary arterial rings, decreases in pulmonary vasoconstrictor responses to ET-1, $BaCl_2$ or KCl were reported in CH rats compared with the controls (Bialecki *et al.*, 1998). However Maclean *et al.* (1995) reported that in extralobar pulmonary arteries from rats with 14 days CH, sensitivity to ET-1 was increased. The pulmonary vessel rings showed discrepancy of vasoconstrictor responses to different agonists during CH. Again, these results suggests that pulmonary vascular hyper-reactivity during pulmonary hypertension is not simply because of pulmonary muscularization.

4.5 Effect of CH on metabolism of catecholamines in isolated lungs

Exposure to hypoxia stimulates sympathetic activity and increases circulating catecholamine levels (Morgan *et al.*, 1995; Heindl *et al.*, 2001). The enhanced vascular reactivity to adrenoceptor agonists, PHE and NA in CH lungs, described before could be due to changes in the number of adrenoceptors or changes in metabolism of catecholamines during CH. So, it was of interest to study the metabolism of catecholamines during CH.

The lung is an important organ which inactivates many biological substances in plasma, for example catecholamines, 5-HT, ET-1 etc. (Vane, 1969; Bryan-Lluka *et al.*, 1992a). So, isolated perfused lungs were used for investigation of catecholamine removal. Similar to other reports (Bakhle & Vane, 1974), this study has shown that the lungs removed almost all 5-HT (82 %) in normoxic rats. NA removal was higher than Adr removal in normoxic lungs. The differential removal of catecholamines in the lungs has been observed by others (Bryan-Lluka & O'Donnell, 1992b). The explanation for this phenomenon could be due to the different affinities of $uptake_1$ and $uptake_2$ for Adr and NA (Trendelenburg, 1991).

Interestingly, CH increased the Adr removal in the lungs, but did not change NA or 5-HT removal. The increase of catecholamine removal is explained as increased sympathetic nerve activity during hypoxia, the increase of catecholamine removal that attenuates the rise of plasma concentration of catecholamine.

A previous study has shown that normoxic perfusion (20% O_2) of CH lungs increased basal PPP, whereas hypoxic perfusion of CH lungs caused no change (Lal *et al.*, 1999b). In order to see any effect of changes in oxygen concentration on catecholamine removal, CH lungs were perfused with Krebs' solution gassed by different oxygen concentrations. Results showed that there was no difference in Adr removal in CH lungs when perfused with normoxic (20%) and hypoxic (10%) mixture.

In normoxic lungs the removal of catecholamine occurs by $Uptake_1$ rather than $Uptake_2$ (Alabaster & Bakle, 1973; Bryan-Lluka *et al.*, 1992b). During CH corticosterone, an $Uptake_2$ inhibitor, had no effect on the removal of Adr in CH lungs. However, cocaine, an $Uptake_1$ inhibitor, reduced Adr removal. This suggests that the uptake mechanism of catecholamines during CH is the same as normoxia. Although early reports have shown that pulmonary catecholamine uptake occurs by a transporter with the properties of both extraneuronal $Uptake_2$ and neuronal $Uptake_1$, it was found that in intact perfused lungs of rats that the transporter in the pulmonary endothelial cells has the same properties as the neuronal NA transporter, $Uptake_1$ (Porzgen *et al.*, 1998).

When comparing uptake of Adr in the absence and presence of an inhibitor, the later measurements were, of necessity carried out at a later time in the experiment and lung weight has started to increase. Thus Adr uptake in lungs with marked pulmonary oedema was compared to normal lungs. Pulmonary oedema did not affect Adr removal.

The possibility exists that the increased Adr uptake into the walls of the pulmonary blood vessels, coupled with increased plasma concentrations in CH (Morgan *et al.*, 1995) would cause vasoconstriction. This together with increased vascular reactivity could contribute to pulmonary hypertension seen in CH. The enhanced vascular reactivity appears to occur at a late stage, probably via sensitization of the myofilaments to Ca^{2+}, possibly via rho kinase. Although ET synthesis is increased in CH lungs and can sensitize pulmonary blood vessels, its role in the vascular hyper-reactivity seen in CH remains equivocal.

4.6 Conclusions

CH induces pulmonary vascular hyper-reactivity, which was demonstrated in the isolated perfused lungs from CH rats, using various vasoconstrictors (PHE, KCl, Ang II, NA, U46619). However, the agonists had discrepant vasoconstrictor responses in the pulmonary resistance arteries from CH rats.

Although it is demonstrated that ET-1 can potentiate the vasoconstrictor (PHE and Ang II) responses via both ET_A and ET_B receptors in the lungs from normoxic rats, ET receptor antagonists and ET converting enzyme had no effect on the potentiation of vasoconstrictor responses in CH lungs. These results do not suggest a role of endogenous ET in pulmonary vascular hyper-reactivity during CH.

From the time-dependent studies of vasconstrictor responses and pulmonary vascular remodelling, the pulmonary vascular remodelling can partly explain the mechanism of pulmonary vascular hyper-reactivity because the time course of the enhanced vasoconstrictor responses to Ang II and KCl was correlated with the time course of pulmonary vascular remodelling during hypoxia and recovery. However, the onset and offset of CH on the vasoconstrictor responses to PHE was different from the responses to Ang II and KCl and did not parallel the process of pulmonary vascular remodelling under the impact of hypoxia and normoxia. It appears some other factors are involved in PHE-induced vasoconstrictor responses in CH lungs.

4.7 Future work

1. Investigation of the distribution of α-adrenoceptors in pulmonary arterial tree in normoxic and CH rats.

2. Investigation of a Rho-kinase inhibitor (e. g. Y27632) on ET-1 sensitization in permeabilized pulmonary artery rings.

3. Quantified measurement of caffeine-induced contraction in the permeabilized vessels and investigation of the role of the IP_3 receptor in Ca^{2+} release in pulmonary and mesenteric arteries.

4. Investigation of phosphorylated myosin light chain in pulmonary vascular smooth muscle in CH rats, using western Blot technique.

5. Changes of intracellular Ca^{2+} release and Ca^{2+} influx in the pulmonary vasculature during CH.

CHAPTER FIVE

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Abbreviations

Adr	adrenaline
Ang II	angiotensin II
ATP	adenosine 5'-triphosphate
ACH	acetycholine
big ET-1	big endothelin-1
BQ123	cyclo(-D-Trp-D-Asp-Pro-D-Val-Leu-)
BQ788	N-cis-2,6-dimethylpiperidinocarbonyl-L-r-MeLeu-D-Trp(COOMe)-D-Nie-Ona
BSA	bovine serum albumin
$[Ca^{2+}]_i$	intracellular calcium concentration
cAMP	3',5'-adenosine monophosphate
cGMP	3',5'-guanosine monophosphate
CICR	Ca^{2+}-induced Ca^{2+} release
DAG	1,2-diacyl-sn-gylcerol
DMSO	dimethylsulphoxide
EL	elastic laminae
ET-1	endothelin-1
ET-2	endothelin-2
ET-3	endothelin-3
ET_A	Endothelin A receptor
ET_B	Endothelin B receptor
ETs	Endothelins

ED_{50}	Dose required evoking 50% of the maximum contraction of an agonist
EGTA	ethylene glycol-bis(b-aminoethylether)-N,N,N',N'-tetra acetic acid
G protein	heterotrimeric guanine nucleotide-binding protein
GTP	guanosine triphosphate
HPV	hypoxic pulmonary vasoconstriction
5-HT	5-hydroxytryptamine
IP_3	inositol 1,4,5-triphosphate
L-NAME	N-nitro L-argine methyl ester
L-NOARG	N-nitro-L-arginine
LW	Lung weight
MIS	mock intracellular solution
NA	noradrenaline
NO	nitric oxide
PGI_2	prostacyclin
PHE	phenylephrine
PIP	pulmonary inflation pressure
PKA	protein kinase A
PKB	protein kinase B
PKC	protein kinase C
PLA2	phospholipase A2
PLC	phospholipase C
PLD	phospholipase D
PPP	pulmonary perfusion pressure

RSS	rat stomach strip
SR	sarcoplasmic reticulum
VSMC	vascular smooth muscle cell

Publications written from this thesis

1. H. Lal, **Q. Yu**, K.I. Williams & B. Woodward: Hypoxia augments conversion of big-endothelin-1 and endothelin ETB receptor-mediated actions in rat lungs. Eur. J. Pharmacol. 402: 101-110, 2000.

2. **Q. Yu**, H. Lal, B. Woodward & K.I. Williams: ET-1 sensitizes actions of vasoconstrictor agents in rat isolated perfused lungs. Br. Pharmacol. J. 133: 232P, 2001.

3. **Q. Yu**, P.I. Mapp, B. Woodward & K.I. Williams: Changes in pulmonary vascular reactivity during and after chronic hypoxia in rats. Br. J. Pharmacol., 2002 (in press).

4. **Q. Yu**, P.I. Mapp, B. Woodward & K.I. Williams: Changes in pulmonary vascular reactivity during chronic hypoxia (in press).

5. **Q. Yu**, B. Woodward & K.I. Williams: The role of ET-1 in vasoconstrictor responses in the isolated perfused lungs of normoxic and chronically hypoxic rats (in press).

Abstract 1

ET-1 sensitizes actions of vasoconstrictor agents in rat isolated perfused lungs

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Previous studies have shown that endothelin-1 (ET-1) can increase the sensitivity of the contractile apparatus to Ca^{2+} in permeabilized rings of rat pulmonary artery (Evans et al., 1999). In the present study we have investigated whether ET-1 can similarly sensitise the pulmonary vascular bed in perfused rat lungs, which contains fine resistance vessels.

Male Wistar rats (280-340g) were anaesthetised with pentobarbital sodium (100 mg/kg, I.P.) and heparinized (500 IU, I.V.). The pulmonary artery was cannulated, perfused with Krebs solution at 37°C, aerated by 20% O_2, 75% N_2 and 5% CO_2 and pulmonary perfusion pressure (PPP) recorded via a pressure transducer (Lal et al., 1994). Lungs were artificially ventilated with room air at 28 strokes/min and equilibrated for 15 min before adding drugs. Preliminary experiments studied the optimal ET-1 concentration (0.3–3 nM) and infusion time (15-60 min) for sensitization. These were found to be ET-1 1nM, the sub-threshold concentration, for 30 min (n=7). Dose-responses to phenylephrine (PHE) and angiotensin II (ANG II) were recorded before and after infusing ET-1 (1nM) for 30 min. Antagonists, which did not affect basal PPP, were infused for 15 minutes before injection of PHE and ANG II. All data shown are mean ± s.e.mean. Data were analysed by unpaired Student's t-test or paired t-test and significance was accepted if $P < 0.05$.

ET-1 shifted PHE and ANG II response-curves to the left (figure 1). Responses to 10 nmoles PHE were increased from 2.96 ± 0.25 mmHg to 3.86 ± 0.45 mmHg ($p<0.05$, n=12). Responses to 10 pmoles ANG II were increased from 0.99 ± 0.17 mmHg to

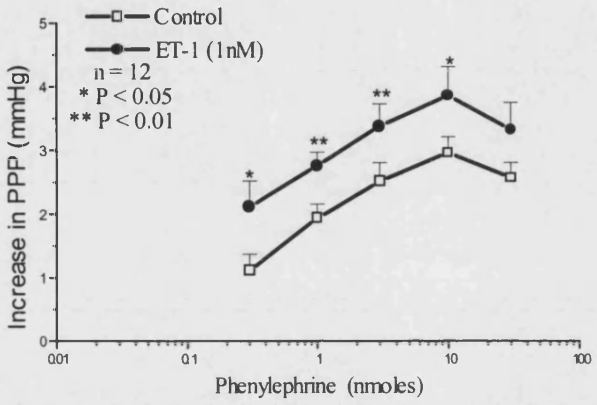

Figure 1: Effect of ET-1 (1nM) on PHE responses in rat isolated perfused lungs.

2.57 ± 0.42 mmHg (n=8, $p<0.05$). Time-matched studies showed that PHE responses repeated after 30 min were not significantly different to controls. The selective ET_A receptor antagonist, PD156707 (5μM) and the selective ET_B receptor antagonist, BQ788 (5μM) reversed the potentiated responses to PHE and ANG II by ET-1. PPP responses to 10 nmoles PHE and 10 pmoles ANG II with ET-1 were reduced by PD156707 (5μM) from 3.86 ± 0.45 mmHg to 1.91 ± 0.26mmHg (n=4-12, $P<0.01$), and 2.57 ± 0.42 mmHg to 0.95 ± 0.24mmHg (n=4-8, $P<0.01$), respectively. BQ788 (5μM) shifted PPP responses to 10 nmoles PHE and 10 pmoles ANG II with ET-1 from 3.86 ± 0.45 mmHg to 2.01 ± 0.57mmHg (n=5-12, $P<0.05$) and 2.57 ± 0.42 mmHg to 0.93 ± 0.33mmHg (n=5-8, $P<0.05$), respectively. These results suggest that ET-1 can sensitize the pulmonary resistance vessels to vasoconstrictors and this action involves both ET_A and ET_B

receptors.

Q. Yu holds an University studentship and ORS award.

Evans et al., (1999) *Br. J. Pharmacol.,* 127, 153-160.

Lal et al., (1994) *Pulm Pharmacol.*, 7, 271-8.

Abstract 2

Changes in pulmonary vascular reactivity during and after chronic hypoxia in rats.

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Chronic hypoxia (CH) induces pulmonary hypertension as a result of vasoconstriction and pulmonary remodelling. The pulmonary vasculature in CH also becomes hyper-responsive to vasoconstrictors. The aim of the present study was to investigate whether enhanced pulmonary vascular reactivity is solely attributable to vascular smooth muscle proliferation.

Male Wistar rats were exposed to normoxia or hypoxia (10% O2) for 3 weeks. For recovery, CH rats were returned to room air and utilised 3 weeks later. Lungs were perfused (Lal *et al.*, 1994). Phenylephrine (PHE) (1-30nmoles), KCl (25-400umoles) and angiotensin II (1-300pmoles) were injected into the pulmonary artery and increases in pulmonary perfusion pressure (PPP) recorded. Weights of the right ventricle (RV) and left ventricle plus septum (LV) were measured for an index of right ventricular hypertrophy. For histology, after fixation with formol saline (0.4%), transverse slices (4μm) of lung tissue were stained with haemotoxylin and eosin. Vessel diameters (range: 37 - 815 μm) and medial wall thickness were measured using a Zeiss KS300 computer image programme. The percentage wall thickness was calculated from the formula: wall thickness (%) = 2× wall thickness × 100/ external diameter (Hislop *et al.*, 1976). Data

were expressed as mean ± s.e.mean and analysed by a one-way ANOVA with Dunnett's test or Student-t test.

Basal PPP increased from 6.3 ± 0.9 mmHg in control to 10.5 ±0.2 mmHg after CH ($P<0.05$, n = 5) but fell again after recovery, (6.4 ± 0.5mmHg, $P>0.05$, n=6). PPP increases to 10nmoles PHE were 2.74 ± 0.28 mmHg in control vs 15.35 ± 3.01 mmHg in CH ($P<0.05$, n=5-7). After 3 weeks recovery, PHE responses had declined (3.08 ± 0.52 mmHg). PPP increases to 200umoles KCl were 8.48 ± 1.20 mmHg in control vs 18.67 ± 3.76 mmHg in CH ($P<0.05$, n=5) and were still elevated after recovery (14.19 ± 1.04 mmHg). This was also true for angiotensin II (100pmoles), 6.83 ± 1.08 mmHg in control vs 17.33 ± 3.74 mmHg in CH ($P<0.05$, n=5-6) and 12.02 ± 1.26 mmHg after recovery. RV/LV ratio was increased from 0.205 ± 0.013 in control to 0.338 ± 0.010 ($P<0.05$) in CH. After recovery the ratio (0.270 ± 0.019) was still significantly elevated ($P<0.05$, n=5-6). Similarly, pulmonary arterial wall thickness was significantly increased from 17.5 ± 1% (n=84) in age-matched control to 21.8 ± 0.9% (n=106) in CH, $P < 0.01$ and was still higher after recovery, 18.4 ± 0.7% (n=114) in age-matched control vs 25.7 ±1% (n=89) in the recovery group, $P<0.001$.

In conclusion, our study shows that CH induces pulmonary hypertension, vascular re-modelling and markedly increases pulmonary vascular reactivity. During recovery from hypoxia, responses to phenylephrine decline faster than those to KCl or angiotensin II. This suggests that in CH proliferation of vascular smooth muscle alone cannot account for the observed vascular hyper-reactivity to α_1-adrenoceptor agonists.

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